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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 45/00, C07K 17/00, C12N 5/00 — A1

(11) International Publication Number: WO 96/27392

(43) International Publication Date: 12 September 1996 (12.09.96)

(21) International Application Number: PCT/US96/03249

(22) International Filing Date: 8 March 1996 (08.03.96)

(30) Priority Data:
08/400,338 — 8 March 1995 (08.03.95) — US

(60) Parent Application or Grant
(63) Related by Continuation
US
Filed on 08/400,338 (CIP)
8 March 1995 (08.03.95)

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(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: ANTIGEN PRESENTING SYSTEM AND METHODS FOR ACTIVATION OF T-CELLS

(57) Abstract

The present invention relates to synthetic antigen-presenting matrices, their methods of making and their methods of use. One such matrix is cells that have been transfected to produce MHC antigen-presenting molecules and assisting molecules such as co-stimulatory molecules. The matrices can be used to activate $CD8^+$ T-cells to produce cytokines and become cytotoxic.

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ANTIGEN PRESENTING SYSTEM AND METHODS FOR ACTIVATION OF T-CELLS

This invention was made with the support of the Government of the United States of America, and the Government of the United States of America has certain rights in the invention.

CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of U.S. patent application Serial No. 08/400,338 filed March 8, 1995 and having the same title.

TECHNICAL FIELD

The present invention relates to materials and methods of activating T-cells with specificity for particular antigenic peptides, the use of activated T-cells *in vivo* for the treatment of a variety of disease conditions, and compositions appropriate for these uses.

BACKGROUND

The efficiency with which the immune system cures or protects individuals from infectious disease has always been intriguing to scientists, as it has been believed that it might be possible to activate the immune system to combat other types of diseases. Such diseases include cancer, AIDS, hepatitis and infectious disease in immunosuppressed patients. While various procedures involving the use of antibodies have been applied in those types of diseases, few if any successful attempts using cytotoxic T-cells have been recorded. Theoretically, cytotoxic T-cells would be the preferable means of treating the types of disease noted above. However, no procedures have been available to specifically activate cytotoxic T-cells.

Cytotoxic T-cells, or $CD8^+$ cells (i.e., cells expressing the molecule CD8) as they are presently known, represent the main line of defense against viral infections. $CD8^+$ lymphocytes specifically recognize and

kill cells which are infected by a virus. Thus, the cost of eliminating a viral infection is the accompanying loss of the infected cells. The T-cell receptors on the surface of $CD8^+$ cells cannot recognize foreign antigens directly. In contrast to antibodies, antigen must first be presented to the receptors.

The presentation of antigen to $CD8^+$ T-cells is accomplished by major histocompatibility complex (MHC) molecules of the Class I type. The major histocompatibility complex (MHC) refers to a large genetic locus encoding an extensive family of glycoproteins which play an important role in the immune response. The MHC genes, which are also referred to as the HLA (human leucocyte antigen) complex, are located on chromosome 6 in humans. The molecules encoded by MHC genes are present on cell surfaces and are largely responsible for recognition of tissue transplants as "non-self". Thus, membrane-bound MHC molecules are intimately involved in recognition of antigens by T-cells.

MHC products are grouped into three major classes, referred to as I, II, and III. T-cells that serve mainly as helper cells express CD4 and primarily interact with Class II molecules, whereas CD8-expressing cells, which mostly represent cytotoxic effector cells, interact with Class I molecules.

Class I molecules are membrane glycoproteins with the ability to bind peptides derived primarily from intracellular degradation of endogenous proteins. Complexes of MHC molecules with peptides derived from viral, bacterial and other foreign proteins comprise the ligand that triggers the antigen responsiveness of T-cells. In contrast, complexes of MHC molecules with peptides derived from normal cellular products play a role in "teaching" the T-cells to tolerate self-peptides,

in the thymus. Class I molecules do not present entire, intact antigens; rather, they present peptide fragments thereof, "loaded" onto their "peptide binding groove".

For many years, immunologists have hoped to raise specific cytotoxic cells targeting viruses, retroviruses and cancer cells. While targeting against viral diseases in general may be accomplished *in vivo* by vaccination with live or attenuated vaccines, no similar success has been achieved with retroviruses or with cancer cells. Moreover, the vaccine approach has not had the desired efficacy in immunosuppressed patients. At least one researcher has taken the rather non-specific approach of "boosting" existing $CD8^+$ cells by incubating them *in vitro* with IL-2, a growth factor for T-cells. However, this protocol (known as LAK cell therapy) will only allow the expansion of those $CD8^+$ cells which are already activated. As the immune system is always active for one reason or another, most of the IL-2 stimulated cells will be irrelevant for the purpose of combatting the disease. In fact, it has not been documented that this type of therapy activates <u>any</u> cells with the desired specificity. Thus, the benefits of LAK cell therapy are controversial at best, and the side effects are typically so severe that many studies have been discontinued.

Several novel molecules which appear to be involved in the peptide loading process have recently been identified. It has also been noted that Class I molecules without bound peptide (i.e., "empty" molecules) can be produced under certain restrictive circumstances. These "empty" molecules are often unable to reach the cell surface, however, as Class I molecules without bound peptide are very thermolabile. Thus, the "empty" Class I molecules disassemble during their transport from the interior of the cell to the cell surface.

The presentation of Class I MHC molecules bound to peptide alone has generally ineffective in activating $CD8^+$ cells. In nature, the $CD8^+$ cells are activated by antigen-presenting cells which present not only a peptide-bound Class I MHC molecule, but also a costimulatory molecule. Such costimulatory molecules include B7 which is now recognized to be two subgroups designated as B7.1 and B7.2. It has also been found that cell adhesion molecules such as integrins assist in this process.

When the $CD8^+$ T-cell interacts with an antigen-presenting cell having the peptide bound by a Class I MHC and costimulatory molecule, the $CD8^+$ T-cell is activated to proliferate and becomes an armed effector T-cell. See, generally, Janeway and Travers, Immunobiology, published by Current Biology Limited, London (1994), incorporated by reference.

Accordingly, what is needed is a means to activate T-cells so that they proliferate and become cytotoxic. It would be useful if the activation could be done *in vitro* and the activated cytotoxic T-cells reintroduced into the patient. It would also be desirable if the activation could be done by a synthetic antigen-presenting matrix comprised of a material such as cells which not only presents the selected peptide, but also presents other costimulatory factors which increase the effectiveness of the activation.

It would also be advantageous if it was possible to select the peptide so that substantially only those $CD8^+$ cells cytotoxic to cells presenting that peptide would be activated.

BRIEF SUMMARY OF THE INVENTION

The present invention relates to a synthetic antigen-presenting system for presenting an MHC molecule

complexed to a peptide and an assisting molecule to a T-cell to activate the T-cell.

In one embodiment, the system relates to a synthetic antigen-presenting matrix having a support and at least the extracellular portion of a Class I MHC molecule capable of binding to a selected peptide operably linked to the support. The matrix also includes an assisting molecule operably linked to the support. The assisting molecule acts on a receptor on the CD8· T-cell. The MHC and assisting molecules are present in sufficient numbers to activate a population of T-cell lymphocytes against the peptide when the peptide is bound to the extracellular portion of the MHC molecule.

It has been found that an antigen-presenting matrix having both an MHC molecule or a portion of a MHC molecule together with an assisting molecule, provides a synergistic reaction in activating T-cell lymphocytes against the peptide. Examples of assisting molecules are costimulatory molecules such as B7.1 and B7.2 or adhesion molecules such as ICAM-1 and LFA-3. The extracellular portion of such costimulatory molecules can also be used. Another type of costimulatory molecule is one that reacts with the CD28 molecule such as anti-CD28 antibodies or functional portions thereof, e.g. Fab portions.

It has been found that a specifically effective synergistic reaction results from an antigen-presenting matrix having MHC molecules bound with a peptide, a costimulatory molecule, and an adhesion molecule. In particular, a highly effective synergistic generation of cytotoxic T-cell activity results from the combination of B7.1 and ICAM-1.

The support used for the matrix can take several different forms. Examples for the support include solid support such as metals or plastics, porous materials such as resin or modified cellulose columns,

microbeads, microtiter plates, red blood cells and liposomes.

Another type of support is a cell fragment, such as a cell membrane fragment or an entire cell. In this embodiment, the matrix is actually cells which have been transfected to present MHC molecules and assisting molecules on the cell surface to create an antigen-presenting cell (APC). This is done by producing a cell line containing at least one expressible Class I MHC nucleotide sequence for the MHC heavy chain, preferably a cDNA sequence, operably linked to a first promoter and an expressible β-2 microglobulin nucleotide sequence operably linked to a second promoter. The MHC heavy chain and the β-2 microglobulin associate together form the MHC molecule which binds to the peptide. The MHC protein binds with the antigenic peptide and presents it on the surface of the cell. The cell also includes a gene for a nucleotide sequence of an assisting molecule operably linked to a third promoter. The assisting molecule is also presented on the surface of the cell. These molecules are presented on the surface of the cell in sufficient numbers to activate a population of T-cell lymphocytes against the peptide when the peptide is bound to the complexes. Other molecules on the surface of a cell or cell fragment such as carbohydrate moieties may also provide some costimulation to the T-cells.

The cell line is synthetic in that at least one of the genes described above is not naturally present in the cells from which the cell line is derived. It is preferable to use a poikilotherm cell line because MHC molecules are thermolabile. A range of species are useful for this purpose. See, for example, U.S. Patent No. 5,314,813 to Petersen et al. which discusses numerous species for this use and is incorporated by reference.

It is preferred to use eukaryotic cells and insect cells in particular.

In one embodiment, it is particularly preferred to have at least two assisting molecules, one being a costimulatory molecule and the other being an adhesion molecule. It has been found that this combination has a synergistic effect, giving even greater T-cell activation than either of the individual molecules combined. It has also been found to be advantageous and preferable to have at least one of the transfected genes under control of an inducible promoter.

Using the present invention, it is possible to introduce the peptide to the cell while it is producing MHC molecules and allow the peptide to bind the MHC molecules while they are still within the cell. Alternatively, the MHC molecules can be expressed as empty molecules on the cell surface and the peptide introduced to the cells after the molecules are expressed on the cell surface. In this latter procedure, the use of poikilotherm cells is particularly advantageous because empty MHC molecules, those not yet complexed or bound with peptides, are thermolabile.

Class I MHC molecules have been expressed in insect cells such as *Drosophila melanogaster* (fruit fly) cells. Since *Drosophila* does not have all the components of a mammalian immune system, the various proteins involved in the peptide loading machinery should be absent from such cells. The lack of peptide loading machinery allows the Class I molecules to be expressed as empty molecules at the cell surface.

Another advantage of using insect cells such as the *Drosophila* system is that *Drosophila* cells prefer a temperature of 28 °C rather than 37 °C. This fact is very important, because empty Class I molecules are thermolabile and tend to disintegrate at 37 °C. By

incubating the Class I-expressing *Drosophila* cells with peptides that can bind to the Class I molecule, it is possible to get virtually every Class I molecule to contain one and the same peptide. The cells are accordingly very different from mammalian cells, where the Class I molecules contain many different types of peptides, most of which are derived from our own, innocuous cellular proteins.

The present invention also relates to methods for producing activated $CD8^+$ cells *in vitro*. One method comprises contacting, *in vitro*, $CD8^+$ cells with one of the antigen-presenting matrices described above for a time period sufficient to activate, in an antigen-specific manner, the $CD8^+$ cells. The method may further comprise (1) separating the activated $CD8^+$ cells from the antigen-presenting matrix; (2) suspending the activated $CD8^+$ cells in an acceptable carrier or excipient; and (3) administering the suspension to an individual in need of treatment. The antigens may comprise native or undegraded proteins or polypeptides, or they may comprise antigenic polypeptides which have been cleaved into peptide fragments comprising at least 8 amino acid residues prior to incubation with the human Class I MHC molecules.

In another variation, the invention relates to methods treating conditions in patients and specifically killing target cells in a human patient. The method comprises (1) obtaining a fluid sample containing resting or naive $CD8^+$ cells from the patient; (2) contacting, *in vitro*, the $CD8^+$ cells with an antigen-presenting matrix for a time period sufficient to activate, in an antigen-specific manner, the $CD8^+$ cells; and (3) administering the activated $CD8^+$ cells to the patient. For example, the use of tumor specific peptides allows for the treatment of tumor related diseases by producing

cytotoxic activated CD8+ T-cells. The invention also relates to the method of treating a medical condition by administration of an antigen-presenting matrix in a suitable suspension. In various embodiments the condition may comprise cancer, tumors, neoplasia, viral or retroviral infection, autoimmune or autoimmune-type conditions. In one embodiment, the method of administering the matrix comprises intravenous injection.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1-3 diagram the construction of expression plasmids pRmHa-2 and pRmHa-3. In Figure 1, pRmHa-2 construction is shown; in Figure 2, pRmHa-3 construction is shown; and in Figure 3, the pRmHa-3 vector is illustrated, showing the restriction, polylinker, promoter, and polyadenylation sites; as well as a site at which a nucleotide sequence may be inserted for expression;

Figures 4 and 5 show peptide-induced thermostabilization of HLA B27 and HLA A2.1 expressed on the surface of *Drosophila* cells by HIV peptides. The mean fluorescence of each cell population is shown plotted against the incubation conditions;

Figure 6 illustrates data from an experiment designed to determine whether insect cells can process antigen and load it onto the Class I molecules, and whether the latter can present either endogenously or exogenously derived antigen to T-cells. Schneider 2 (SC2) or 3T3 cells transfected with $K^b/\beta 2$ were incubated with ovalbumin protein (OvPro) or ovalbumin peptide, OVA 24 (OvPep) in isotonic (Iso) or hypertonic (Hyp) media. (Murine cell line BALB/3T3 is available from the ATCC under accession number CCL 163.) After treatment, cells were cocultured with the T-cell hybridoma B3/CD8. B3/CD8 is a T-cell hybridoma between B3 (Carbone, et al., J. Exp. Med. 169: 603-12 (1989)), cytotoxic T-cell specific

for ovalbumin peptide 253-276 presented by H-2K^b Class I molecules, and CD8- bearing IL-2-secreting cell line. Upon antigenic stimulation, B3/CD8 produces IL-2, measured by ^{3}H-thymidine incorporation in IL-2-dependent cell line CTLL (Gillis, et al., J. Immunol. 120: 2027 91978)). Thus, by measuring the amount of IL-2 produced, one can assay for T-cell recognition. The supernatant from the cocultures were analyzed for IL-2 by ^{3}H thymidine incorporation by the IL-2-dependent cell line CTLL (ATCC No. TIB 214). The amount of ^{3}H thymidine incorporated is plotted against the initial cell treatments;

Figure 7 illustrates the expression of B7.1, ICAM-1 and MHC on the surface of transfected *Drosophila* (fly) cells according to the present invention;

Figure 8 is a graph showing results of a fluorescence-activated cell sorter experiment using recombinant L^d mouse MHC linked to red blood cells;

Figure 9 is a graph showing results of a fluorescence-activated cell sorter experiment using recombinant K^b mouse MHC linked to red blood cells;

Figure 10 is a graph demonstrates binding of recombinant K^b to microtiter plates by use of labeled antibodies;

Figure 11 is a series of graphs showing the results from fluorescence-activated cell sorter experiments demonstrating the expression of CD69 and CD25 on CD8$^+$ 2C cells stimulated with transfected *Drosophila* cells;

Figure 12 is a pair of bar graphs showing IL-2-dependent proliferative responses of CD8$^+$ 2C cells elicited by peptides presented by *Drosophila* cells transfected with L^d only;

Figure 13 is a graph showing the influence of peptide concentration on day 3 proliferative responses of

CD8+ 2C cells elicited by peptides presented by transfected *Drosophila* cells;

Figure 14 is a pair of graphs showing the influence of antigen-presenting cells dose on day 3 proliferative response and IL-2 production of CD8+ 2C cells elicited by peptides presented by transfected *Drosophila* cells;

Figure 15 is a series of graphs showing the influence of peptide concentration on the proliferative response of CD8+ 2C cells elicited by *Drosophila* cells transfected with L^d.B7, L^d.ICAM and L^d.B7.ICAM;

Figure 16 is a series of graphs showing the kinetics of the proliferative response of CD8+ and CD8- 2C cells elicited by *Drosophila* cells transfected with L^d, L^d.B7, L^d.ICAM and L^d.B7.ICAM plus QL9 peptide;

Figure 17 is a series of graphs showing CTL activity of CD8+ 2C cells stimulated by *Drosophila* cells transfected with L^d.B7, L^d.B7.ICAM or L^d.ICAM antigen-presenting cells plus QL9 peptide (10 μM) in the absence of exogenous cytokines;

Figure 18 is a pair of graphs showing CTL activity of CD8+ 2C cells stimulated by *Drosophila* cells transfected with L^d.ICAM antigen-presenting cells plus QL9 peptide (10 μM) in the absence (left) or presence (right) of exogenous IL-2 (20u/ml);

Figure 19 is a pair of graphs showing the proliferative response of normal (non-transgenic) CD8+ T-cells to peptides presented by transfected *Drosophila* cells (left panel) and the response elicited by graded doses of N B6 and 2C B6 CD8+ cells cultured with 5 x 10^4 B10.D2 (L^d) spleen cells (2000 cGy) in the absence of peptides for 3 days without the addition of exogenous cytokines (right panel);

Figure 20 is a graph showing stimulated mitogenesis of purified 2C+ T-cells cultured (50,000

cells per well) in plates coated with immobilized molecules with peptide QL9 (solid line = L^d and anti-CD28 antibody, broken line = L^d only);

Figure 21 is a bar graph showing stimulated mitogenesis of purified 2C+ T-cells at day 5 in culture, with various indicated peptides, cultured in 96-well plates coated with L^d and anti-CD28 antibody (hatched bars = no IL-2, black bars = IL-2 added);

Figure 22 is a graphical representation of the results of cytofluorometric analysis of cells recovered after 12 days of culture in plates coated with L^d and anti-CD28 antibody and exposed to peptide QL9, stained using the 2C T-cell receptor specific antibody 1B2 (M2 = positive staining cells, M1 = negative staining cells);

Figure 23 is a graphical representation of the results of cytofluorometric analysis of cells recovered after 12 days of culture in plates coated with L^d and anti-CD28 antibody and exposed to peptide p2Ca, stained using the 2C T-cell receptor specific antibody 1B2 (M2 = positive staining cells, M1 = negative staining cells);

Figure 24 is a graphical representation of the results of cytofluorometric analysis of cells recovered after 12 days of culture in plates coated with L^d and anti-CD28 antibody and exposed to peptide SL9, stained using the 2C T-cell receptor specific antibody 1B2 (M2 = positive staining cells, M1 = negative staining cells);

Figure 25 is a graph showing cytolysis of target cells by activated T-cells (solid line = peptide QL9, broken line = control peptide LCMV);

Figure 26 is a graphical representation of the cytotoxic lysis resulting from activation of human CD8+ T-cells with antigen-presenting cells loaded with influenza matrix peptide;

Figure 27 is a graphical representation of the cytotoxic lysis resulting from activation of human CD8+

T-cells with antigen-presenting cells loaded with HIV-RT peptide; and

Figure 28 is a graphical representation of the cytotoxic lysis resulting from activation of human CD8· T-cells with antigen-presenting cells loaded with tyrosinase peptide.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a synthetic antigen-presenting system which can be used to activate T-cell lymphocytes. The activated CD8· T-cells proliferate, produce cytokines, become cytotoxic or some combination of these results. In one preferred embodiment, the system activates cytotoxic CD8· cells which then proliferate and then are activated to seek out and destroy target cells. The present invention can be used to activate T-cells *in vitro* and the activated T-cells are then returned to the patient from which they were originally derived or may be used *in vivo* activation of T-cells.

The synthetic antigen-presenting system of the present invention has two major components. The first component is at least the extracellular portion of the Class I MHC molecule which is capable of binding to a selected peptide. The second major component is an assisting molecule which assists in the activation of T-cells. In each case, an extracellular portion of a larger molecule can used, but in certain embodiments, the entire molecules are used.

For ease of description, MHC molecules will be discussed generally, with the understanding that an extracellular portion of the MHC molecule may be used. The portion of the MHC molecule necessary for the present invention is the part which binds to the selected peptide and presents the peptide to the T-cell.

The peptide is selected to activate the appropriate T-cell, depending on the treatment to be conducted. For example, in the treatment of particular cancers, certain antigenic peptides are presented on the surface of the cancer cells which will react with activated T-cells. Thus, it is appropriate to use a peptide selected to activate the appropriate T-cells that will then bind with and destroy the cancer cells.

The present invention allows the MHC molecules to be produced by cells with the peptide already complexed with the MHC molecule or to produce empty MHC molecules which do not yet have a peptide complexed with them. This latter embodiment is particularly useful since it allows the peptide to be chosen after the MHC molecules are prepared.

A Class I MHC molecule includes a heavy chain, sometimes referred to as an alpha chain, and a β-2 microglobulin. As discussed herein, the extracellular portion of the Class I MHC molecule is made up of an extracellular portion of an MHC heavy chain together with the β-2 microglobulin.

In preparing the extracellular portions of MHC to be linked to a support, soluble molecules are prepared as discussed below. These molecules generally lack the transmembrane and cytoplasmic domain in the MHC molecule.

The assisting molecule helps facilitate the activation of the T-cell when it is presented with a peptide/MHC molecule complex. The present invention includes two major categories of assisting molecules. The first category is composed of costimulatory molecules such as B7.1 (previously known as B7 and also known as CD80) and B7.2 (also known as CD86) which binds to CD28 on T-cells. Other costimulatory molecules are anti-CD28 antibodies or the functional portions of such antibodies, e.g. Fab portions that bind to CD28.

The other major category of assisting molecules of the present invention are adhesion molecules. These include the various ICAM molecules, which include ICAM-1, ICAM-2, ICAM-3 and LFA-3. It has been found that the combination of a peptide bound to an MHC molecule used in conjunction with one of these assisting molecules activates the T-cells to an extent previously not seen.

An even greater synergistic reaction has been achieved by using a peptide-bound MHC molecule in conjunction with both a costimulatory molecule and an adhesion molecule. This has been found to be particularly effective in producing cytotoxic $CD8^+$ cells.

In accordance with the present invention, the MHC molecule and the assisting molecule are operably linked to a support such that the MHC and assisting molecules are present in sufficient numbers to activate a population of T-cells lymphocytes against the peptide when the peptide is bound to the extracellular portion of the MHC molecule. The peptide can be bound to the MHC molecule before or after the MHC molecule is linked to the support.

The support can take on many different forms. It can be a solid support such as a plastic or metal material, it can be a porous material such as commonly used in separation columns, it can be a liposome or red blood cell, or it can even be a cell or cell fragment. As discussed in more detail below, in the case where a cell serves as a support, the MHC and assisting molecules can be produced by the cell. The MHC molecule is then linked to the cell by at least the transmembrane domain if not also the cytoplasmic domain which would not be present in a soluble form of MHC.

The extracellular portions of MHC molecule and assisting molecule can be linked to a support by providing an epitope which reacts to an antibody

immobilized on the support. In addition, the MHC or assisting molecules can be produced with or linked to $(His)_6$ which would react with nickel in forming part of the support. Other means to immobilize or link MHC molecules to a support are well known in the art.

As discussed above, the support can be a cell membrane or an entire cell. In such a case, an eukaryotic cell line is modified to become a synthetic antigen-presenting cell line for use with T-cell lymphocytes. For ease of description, antigen-presenting cells (APC) will also be called stimulator cells. Because empty MHC molecules are thermolabile, it is preferred that the cell culture be poikilotherm and various cell lines are discussed in detail below.

A culture of cells is first established. The culture is then transfected with an expressible Class I MHC heavy chain gene which is operably linked to a promoter. The gene is chosen so that it is capable of expressing the Class I MHC heavy chain. The cell line is also transfected with an expressible β-2 microglobulin gene which is operably linked to a second promoter. The gene is chosen such it is capable of expressing β-2 microglobulin that forms MHC molecules with the MHC heavy chain. In the case of soluble extracellular portions of MHC molecules to be used with solid supports and the like, a truncated MHC heavy chain gene is used as discussed in more detail below.

The culture is also transfected with an expressible assisting molecule gene operably linked to a third promoter. The assisting molecule gene is capable of being expressed as an assisting molecule which interacts with the molecule on the T-cell lymphocytes. As discussed below, each of these genes can be transfected using various methods, but the preferred method is to use more than one plasmid.

The cell line transfected is chosen because it lacks at least one of the genes being introduced. It has been found that insect cells are advantageous not only because they are poikilothermic, but because they lack these genes and the mechanisms which would otherwise produce MHC molecules bound to peptides. This allows for greater control over the production of peptide-bound MHC molecules, and the production of empty MHC molecules. The MHC heavy chain is preferably from a different species, more preferably, a homeotherm such as mammals and, optimally, humans.

The preferred cell line is a stable poikilotherm cell line that has the first promoter being inducible to control the expression of the MHC heavy chain. It is preferred that the cell assembles empty MHC molecules and presents them on the cell surface so that the peptides can be chosen as desired.

The resulting MHC molecules bind to the peptide and are present in sufficient numbers with the assisting molecules on the surface of the cell to activate a population of T-cell lymphocytes against the peptide when the peptide is bound to the MHC cells.

In a further embodiment, a second assisting molecule gene is also transfected into the cell culture. In this case, the first assisting molecule gene can be for a costimulatory molecule and the second assisting molecule gene can be for an adhesion molecule.

It is preferred that at least one of the genes and, in particular, the MHC heavy chain gene be linked to an inducible promoter. This allows control over the production of MHC molecules so that they are only produced at a time when the peptide of interest is available and presented in the culture to react with the produced MHC molecules. This minimizes undesirable MHC molecule/peptide complexes.

Where the cell line already produces one or more of the desired molecules, it is only necessary to transfect the culture with an expressible gene for the gene which is lacking in the cells. For example, if the cells already present the MHC molecules on their surface, it is only necessary to transfect the culture with an expressible gene for the assisting molecule.

The peptide can be introduced into the cell culture at the time the cells are producing MHC molecules. Through methods such as osmotic shock, the peptides can be introduced in the cell and bind to the produced MHC molecules. Alternatively, particularly in the case poikilotherm cell lines, the MHC molecules will be presented empty on the cell surface. The peptide can then be added to the culture and bound to the MHC molecules as desired.

After the cells are produced having MHC and assisting molecules on their surfaces, they can be lyophilized and the fragments of the cells used to activate the population of T-cell lymphocytes.

Transfected cultures of cells can be used to produced extracellular portions of MHC molecules and assisting molecules. The use of extracellular portions in conjunction with supports such as solid supports has certain advantages of production. Where living cells are used to provide a synthetic antigen-presenting cell, at least three genes, two to produce the MHC molecule and one for the assisting molecule must be introduced to the cell. Often, additional genes such as for antibiotic resistance are also transfected.

Where a solid support system is being used, one cell line can produce the extracellular portions of MHC molecules while another cell line produces the extracellular portion of the assisting molecule. The MHC molecule portions and the assisting molecule portions can

then be harvested from their respective cultures. The molecules are then linked to an appropriate support in sufficient numbers to activate a population of T-cell lymphocytes against a peptide when the peptide is bound to the extracellular portion of the MHC molecule. From a production standpoint, two different cultures can be used, but it is also possible to use the same culture, however, requiring that the culture be transfected with the additional gene for the extracellular portion of the assisting molecule.

A further modification of this embodiment is to provide a third culture of cells which is transfected with an expressible second assisting molecule gene. In this example, the second culture of cells produces extracellular portions of the costimulatory molecule while the third culture of cells produce an extracellular portion of an adhesion molecule. The adhesion molecule portions are harvested and linked to the support.

The present invention also relates to a method for activating $CD8^+$ T-cells against a selected peptide. The method relates to providing a cell line presenting MHC molecules binding a peptide and assisting molecules on their surfaces. Naive $CD8^+$ T-cells can be obtained by removal from a patient to be treated. The cultured cells are then contacted with the $CD8^+$ T-cells for a sufficient period of time to activate the $CD8^+$ T-cell lymphocytes resulting in proliferation and transforming the T-cells into armed effector cells.

The activated $CD8^+$ T-cells can then be separated from the cell line and put into a suspension in an acceptable carrier and administered to the patient. An alternative method involves the use of the synthetic antigen-presenting matrix to activate the $CD8^+$ cells.

It is preferred that human genes are used and, therefore, human molecule analogs are produced. As shown

in prior U.S. Patent No. 5,314,813, murine systems provide particularly useful models for testing the operation of T-cell activation and demonstrate the applicability of the process for human systems. See also Sykulev et al., Immunity 1: 15-22 (1994).

1. Human Class I MHC Molecules

Class I MHC molecules comprise a heavy chain and a β-microglobulin protein. A human Class I MHC heavy chain of the present invention is selected from the group comprising HLA-A, HLA-B, HLA-C, HLA-E, HLA-F, and HLA-G, and more preferably, from the group comprising HLA-A, HLA-B, and HLA-C. The heavy chains are useful in either soluble or insoluble form. In the soluble ("sol") form, a stop codon is engineered into the nucleotide sequence encoding the HLA molecule of choice preceding the transmembrane domain.

While it is possible to isolate nucleotide sequences encoding human Class I MHC heavy chains from known, established cell lines carrying the appropriate variants -- e.g., transformed cell lines JY, BM92, WIN, MOC, and MG -- it is more practical to synthesize the nucleotide sequence from a portion of the gene via polymerase chain reaction (PCR), using the appropriate primers. This method has been successfully used to clone full-length HLA cDNA; for example, the sequences for HLA-A25, HLA-A2, HLA-B7, HLA-B57, HLA-B51, and HLA-B37 are deposited in the GenBank database under accession nos. M32321, M32322, M32317, M32318, M32319 and M32320, respectively. Known, partial and putative HLA amino acid and nucleotide sequences, including the consensus sequence, are published (see, e.g., Zemmour and Parham, Immunogenetics 33: 310-320 (1991)), and cell lines expressing HLA variants are known and generally available as well, many from the American Type Culture Collection ("ATCC"). Therefore, using PCR, it is possible to

synthesize human Class I MHC-encoding nucleotide sequences which may then be operatively linked to a vector and used to transform an appropriate cell and expressed therein.

Particularly preferred methods for producing the Class I MHC heavy chain, β-2 microglobulin proteins and assisting molecules of the present invention rely on the use of preselected oligonucleotides as primers in a polymerase chain reaction (PCR) to form PCR reaction products as described herein. Gene preparation is typically accomplished by primer extension, preferably by primer extension in a polymerase chain reaction (PCR) format.

If the genes are to be produced by (PCR) amplification, two primers, i.e., a PCR primer pair, must be used for each coding strand of nucleic acid to be amplified. (For the sake of simplicity, synthesis of an exemplary HLA heavy chain variant sequence will be discussed, but it is expressly to be understood that the PCR amplification method described is equally applicable to the synthesis of β-2 microglobulin, costimulatory molecules, adhesion molecules, and all HLA variants, including those whose complete sequences are presently unknown.)

The first primer becomes part of the antisense (minus or complementary) strand and hybridizes to a nucleotide sequence conserved among HLA (plus or coding) strands. To produce coding DNA homologs, first primers are therefore chosen to hybridize to (i.e. be complementary to) conserved regions within the MHC genes, preferably, the consensus sequence or similar, conserved regions within each HLA group -- i.e., consensus sequences within HLA-A, HLA-B, HLA-C, and the less-polymorphic groups, HLA-E, -F, and -G.

Second primers become part of the coding (plus) strand and hybridize to a nucleotide sequence conserved among minus strands. To produce the HLA-coding DNA homologs, second primers are therefore chosen to hybridize with a conserved nucleotide sequence at the 5' end of the HLA-coding gene such as in that area coding for the leader or first framework region. In the amplification of the coding DNA homologs the conserved 5' nucleotide sequence of the second primer can be complementary to a sequence exogenously added using terminal deoxynucleotidyl transferase as described by Loh et al., Science 243: 217-220 (1989). One or both of the first and second primers can contain a nucleotide sequence defining an endonuclease recognition site. The site can be heterologous to the immunoglobulin gene being amplified and typically appears at or near the 5' end of the primer.

The high turn over rate of the RNA polymerase amplifies the starting polynucleotide as has been described by Chamberlin et al., The Enzymes, ed. P. Boyer, PP. 87-108, Academic Press, New York (1982). Another advantage of T7 RNA polymerase is that mutations can be introduced into the polynucleotide synthesis by replacing a portion of cDNA with one or more mutagenic oligodeoxynucleotides (polynucleotides) and transcribing the partially-mismatched template directly as has been previously described by Joyce et al., Nuc. Acid Res. 17: 711-722 (1989). Amplification systems based on transcription have been described by Gingeras et al., in PCR Protocols, A Guide to Methods and Applications, pp 245-252, Academic Press, Inc., San Diego, CA (1990).

PCR amplification methods are described in detail in U.S. Patent Nos. 4,683,192, 4,683,202, 4,800,159, and 4,965,188, and at least in several texts including "PCR Technology: Principles and Applications

for DNA Amplification", H. Erlich, ed., Stockton Press, New York (1989); and "PCR Protocols: A Guide to Methods and Applications", Innis et al., eds., Academic Press, San Diego, California (1990). Various preferred methods and primers used herein are described hereinafter and are also described in Nilsson, et al., Cell 58: 707 (1989), Ennis, et al., PNAS USA 87: 2833-7 (1990), and Zemmour, et al., Immunogenetics 33: 310-20 (1991), for example. In particular, it is preferred to design primers from comparison of 5' and 3' untranslated regions of HLA alleles (e.g., -A, -B, -C, -E, -F, or -G alleles), with selection of conserved sequences. Restriction sites may also be incorporated into the 5' and 3' primers to enable the amplification products to be subcloned into sequencing or expression vectors. It may also be helpful to place a 4-base spacer sequence proximal to the restriction site to improve the efficiency of cutting amplification products with enzymes.

The following primers are preferred for amplification of HLA-A, -B, -C, -E, -F, and -G cDNA, preferably in separate reactions. Resulting cDNAs may then be cloned and sequenced as described herein. These primers are appropriate for use in amplifying all known and presently unknown types of HLA.

HLA A

5' primer: 5' CC ACC ATG GCC GTC ATG GCG CCC 3'

(SEQ ID NO 1)

3' primer: 5' GG TCA CAC TTT ACA AGC TCT GAG 3'

(SEQ ID NO 2)

HLA B

5' primer: 5' CC ACC ATG CTG GTC ATG GCG CCC 3'

(SEQ ID NO 3)

3' primer: 5' GG ACT CGA TGT GAG AGA CAC ATC 3'

(SEQ ID NO 4)

HLA C

5' primer: 5' CC ACC ATG CGG GTC ATG GCG CCC 3'

(SEQ ID NO 5)

3' primer: 5' GG TCA GGC TTT ACA AGC GAT GAG 3'

(SEQ ID NO 6)

HLA E

5' primer: 5' CC ACC ATG CGG GTA GAT GCC CTC C 3'

(SEQ ID NO 7)

3' primer: 5' GG TTA CAA GCT GTG AGA CTC AGA 3'

(SEQ ID NO 8)

HLA F

5' primer: 5' CC ACC ATG GCG CCC CGA AGC CTC 3'

(SEQ ID NO 9)

3' primer: 5' GG TCA CAC TTT ATT AGC TGT GAG A 3'

(SEQ ID NO 10)

HLA G

5' primer: 5' CC ACC ATG GCG CCC CGA ACC CTC 3'

(SEQ ID NO 11)

3' primer: 5' GG TCA CAA TTT ACA AGC CGA GAG 3'

(SEQ ID NO 12)

In preferred embodiments only one pair of first and second primers is used per amplification reaction. The amplification reaction products obtained from a

plurality of different amplifications, each using a plurality of different primer pairs, are then combined. However, the present invention also relates to DNA homolog production via co-amplification (using two pairs of primers), and multiplex amplification (using up to about 8, 9 or 10 primer pairs).

In preferred embodiments, the PCR process is used not only to produce a variety of human Class I-encoding DNA molecules, but also to induce mutations which may emulate those observed in the highly-polymorphic HLA loci, or to create diversity from a single parental clone and thereby provide a Class I MHC molecule-encoding DNA "library" having a greater heterogeneity. In addition to the mutation inducing variations described in the above referenced U.S. Patent No. 4,683,195 and such as discussed in U.S. Patent No. 5,314,813.

2. DNA Expression Vectors

A vector of the present invention is a nucleic acid (preferably DNA) molecule capable of autonomous replication in a cell and to which a DNA segment, e.g., gene or polynucleotide, can be operatively linked so as to bring about replication of the attached segment. One of the nucleotide segments to be operatively linked to vector sequences encodes at least a portion of a mammalian Class I MHC heavy chain. Preferably, the entire peptide-coding sequence of the MHC heavy chain is inserted into the vector and expressed; however, it is also feasible to construct a vector which also includes some non-coding MHC sequences as well. Preferably, non-coding sequences of MHC are excluded. Alternatively, a nucleotide sequence for a soluble ("sol") form of an Class I MHC heavy chain may be utilized; the "sol" form differs from the non-sol form in that it contains a "stop" codon inserted at the end of the alpha 3 domain or

prior to the transmembrane domain. Another preferred vector includes a nucleotide sequence encoding at least a portion of a mammalian β-2 microglobulin molecule operatively linked to the vector for expression. Still another preferred vector includes a nucleotide sequence encoding at least a portion of a mammalian assisting molecule operably linked to the vector for expression. It is also feasible to construct a vector including nucleotide sequences encoding a Class I MHC heavy chain and a β-2 microglobulin and an assisting molecule, or some combination of these.

A preferred vector comprises a cassette that includes one or more translatable DNA sequences operatively linked for expression via a sequence of nucleotides adapted for directional ligation. The cassette preferably includes DNA expression control sequences for expressing the polypeptide or protein that is produced when a translatable DNA sequence is directionally inserted into the cassette via the sequence of nucleotides adapted for directional ligation. The cassette also preferably includes a promoter sequence upstream from the translatable DNA sequence, and a polyadenylation sequence downstream from the mammalian MHC heavy chain sequence. The cassette may also include a selection marker, albeit it is preferred that such a marker be encoded in a nucleotide sequence operatively linked to another expression vector sequence.

The choice of vector to which a cassette of this invention is operatively linked depends directly, as is well known in the art, on the functional properties desired, e.g., vector replication and protein expression, and the host cell to be transformed, these being limitations inherent in the art of constructing recombinant DNA molecules.

In various embodiments, a vector is utilized for the production of polypeptides useful in the present invention, including MHC variants and antigenic peptides. Exemplary vectors include the plasmids pUC8, pUC9, pUC18, pBR322, and pBR329 available from BioRad Laboratories (Richmond, CA), pPL and pKK223 available from Pharmacia (Piscataway, NJ), and pBS and M13mp19 (Stratagene, La Jolla, CA). Other exemplary vectors include pCMU (Nilsson, et al., Cell 58: 707 (1989)). Other appropriate vectors may also be synthesized, according to known methods; for example, vectors pCMU/K" and pCMUII used in various applications herein are modifications of pCMUIV (Nilsson, et al., *supra*).

In addition, there is preferably a sequence upstream of the translatable nucleotide sequence encoding a promoter sequence. Preferably, the promoter is conditional (e.g., inducible). A preferred conditional promoter used herein is a metallothionein promoter or a heat shock promoter.

Vectors may be constructed utilizing any of the well-known vector construction techniques. Those techniques, however, are modified to the extent that the translatable nucleotide sequence to be inserted into the genome of the host cell is flanked "upstream" of the sequence by an appropriate promoter and, in some variations of the present invention, the translatable nucleotide sequence is flanked "downstream" by a polyadenylation site. This is particularly preferred when the "host" cell is an insect cell and the nucleotide sequence is transmitted via transfection. Transfection may be accomplished via numerous methods, including the calcium phosphate method, the DEAE-dextran method, the stable transfer method, electroporation, or via the liposome mediation method. Numerous texts are available which set forth known transfection methods and other

procedures for introducing nucleotides into cells; see, e.g., Current Protocols in Molecular Biology, John Wiley & Sons, NY (1991).

The vector itself may be of any suitable type, such as a viral vector (RNA or DNA), naked straight-chain or circular DNA, or a vesicle or envelope containing the nucleic acid material and any polypeptides that are to be inserted into the cell. With respect to vesicles, techniques for construction of lipid vesicles, such as liposomes, are well known. Such liposomes may be targeted to particular cells using other conventional techniques, such as providing an antibody or other specific binding molecule on the exterior of the liposome. See, e.g., A. Huang, et al., J. Biol. Chem. 255: 8015-8018 (1980). See, e.g., Kaufman, Meth. Enzymol. 185: 487-511 (1990).

In a preferred embodiment, the vector also contains a selectable marker. After expression, the product of the translatable nucleotide sequence may then be purified using antibodies against that sequence. One example of a selectable marker is neomycin resistance. A plasmid encoding neomycin resistance, such as phshsneo, phsneo, or pcopneo, may be included in each transfection such that a population of cells that express the gene(s) of choice may be ascertained by growing the transfectants in selection medium.

A preferred vector for use according to the present invention is a plasmid; more preferably, it is a high-copy-number plasmid. It is also desirable that the vector contain an inducible promoter sequence, as inducible promoters tend to limit selection pressure against cells into which such vectors (which are often constructed to carry non-native or chimeric nucleotide sequences) have been introduced. It is also preferable that the vector of choice be best suited for expression

in the chosen host. If the host cell population is a *Drosophila* cell culture, then a compatible vector includes vectors functionally equivalent to those such as p25-lacZ (see Bello and Couble, Nature 346: 480 (1990)) or pRmHa-1, -2, or -3 (see Bunch, et al., Nucl. Acids Res. 16: 1043-1061 (1988)). In the preferred embodiment, the vector is pRmHa-3, which is shown in Figure 3. This vector includes a metallothionein promoter, which is preferably upstream of the site at which the MHC sequence is inserted, and the polyadenylation site is preferably downstream of said MHC sequence. Insect cells and, in particular, *Drosophila* cells are preferred hosts according to the present invention. *Drosophila* cells such as Schneider 2 (S2) cells have the necessary trans-acting factors required for the activation of the promoter and are thus even more preferred.

The expression vector pRmHa-3 is based on the bacterial plasmid pRmHa-1 (Figure 2), the latter of which is based on plasmid pUC18 and is deposited with the American Type Culture Collection (ATCC, Rockville, MD), having the accession number 37253. The pRmHa-3 vector contains the promoter, the 5' untranslated leader sequence of the metallothionein gene (sequences 1-421, SEQ ID NO 13) with the R1 and Stu sites removed; see Figure 3). It also contains the 3' portion of the *Drosophila* ADH gene (sequence #6435-7270, SEQ ID NO 14) including the polyadenylation site. Therefore, cloned DNA will be transcriptionally regulated by the metallothionein promoter and polyadenylated.

Construction of the pRmHa-1 plasmid is described in Bunch, et al., Nucl. Acids Res. 16: 1043-1061 (1988). Construction of the pRmHa-3 and pRmHa-2 plasmids (the latter of which has a metallothionein promoter sequence that may be removed as an Eco RI fragment) is illustrated in Figures 1, 2, and 3. With regard to pRmHa-3, a

preferred plasmid for use according to the present invention, Pst I, Sph I and Hind III are in the promoter fragment and therefore are not unique. Xba is in the ADH fragment (4 bases from its 3' end) and is also not unique. The following restriction sites are, however, unique in pRmHa-3: Eco RI, Sac I, Kpn I, Sma I, Bam HI, Sal I, Hinc 2, and Acc I.

A cassette in a DNA expression vector of this invention is the region of the vector that forms, upon insertion of a translatable DNA sequence, a sequence of nucleotides capable of expressing, in an appropriate host, a fusion protein of this invention. The expression-competent sequence of nucleotides is referred to as a cistron. Thus, the cassette preferably comprises DNA expression control elements operatively linked to one or more translatable DNA sequences. A cistron is formed when a translatable DNA sequence is directionally inserted (directionally ligated) between the control elements via the sequence of nucleotides adapted for that purpose. The resulting translatable DNA sequence, namely the inserted sequence, is, preferably, operatively linked in the appropriate reading frame.

DNA expression control sequences comprise a set of DNA expression signals for expressing a structural gene product and include both 5' and 3' elements, as is well known, operatively linked to the cistron such that the cistron is able to express a structural gene product. The 5' control sequences define a promoter for initiating transcription and a ribosome binding site operatively linked at the 5' terminus of the upstream translatable DNA sequence.

Thus, a DNA expression vector of this invention provides a system for cloning translatable DNA sequences into the cassette portion of the vector to produce a

cistron capable of expressing a fusion protein of this invention.

3. Cell Lines

A preferred cell line of the present invention is capable of continuous growth in culture and capable of expressing mammalian Class I MHC molecules and assisting molecules on the surface of its cells. Any of a variety of transformed and non-transformed cells or cell lines are appropriate for this purpose, including bacterial, yeast, insect, and mammalian cell lines. (See, e.g., Current Protocols in Molecular Biology, John Wiley & Sons, NY (1991), for summaries and procedures for culturing and using a variety of cell lines, e.g., *E. coli* and *S. cerevisiae*.)

Preferably, the cell line is a eukaryotic cell line. More preferably, the cell line is poikilothermic (i.e., less sensitive to temperature challenge than mammalian cell lines). More preferably, it is an insect cell line. Various insect cell lines are available for use according to the present invention, including moth (ATCC CCL 80), armyworm (ATCC CRL 1711), mosquito larvae (ATCC lines CCL 125, CCL 126, CRL 1660, CRL 1591, CRL 6585, CRL 6586) and silkworm (ATCC CRL 8851). In a preferred embodiment, the cell line is a *Drosophila* cell line such as a Schneider cell line (see Schneider, J. Embryol. Exp. Morph. 27: 353-365 (1972)); preferably, the cell line is a Schneider 2 (S2) cell line (S2/M3) adapted for growth in M3 medium (see Lindquist, et al., *Drosophila* Information Service 58: 163 (1982)).

Schneider cells may be prepared substantially as follows. *Drosophila melanogaster* (Oregon-R) eggs are collected over about a 4 hour interval and are dechlorinated in 2.5% aqueous sodium hypochlorite and surface-sterilized by immersion in 70% ethanol for 20 minutes, followed by an additional 20 minutes in 0.05%

$HgCl_2$ in 70% ethanol. After being rinsed thoroughly in sterile distilled water, the eggs are transferred to petri dishes containing sterile Metricel black filters backed with Millipore prefilters, both previously wetted with culture medium. The eggs are placed overnight in a 22 °C incubator and removed for culturing when 20-24 hours old. The embryos are each cut into halves or thirds, then placed in 0.2% trypsin (1:250, Difco) in Rinaldini's salt solution (Rinaldini, Nature (London) 173: 1134-1135 (1954)) for 20-45 minutes at room temperature. From 100-300 embryos are used to initiate each culture.

After the addition of fetal bovine serum (FBS), the fragments are centrifuged at 100 X g for 2-3 minutes, resuspended in 1.25ml culture medium and seeded into glass T-9 flasks. The cultures are maintained at about 22-27 °C ± 0.5 °C, with a gaseous phase of ambient air. Schneider's culture medium (Schneider, J. Exp. Zool. 156: 91-104 (1964); Schneider, J. Embryol. Exp. Morph. 15: 271-279 (1966)) containing an additional 500mg bacteriological peptone per 100ml medium and supplemented with 15% inactivated FBS is preferably used. The pH (preferably 6.7-6.8) is monitored with 0.01% phenol red. The cell lines are preferably maintained by subculturing every 3-7 days. The cells readily attach to the glass but not so firmly as to require trypsin treatment; typically, simple pipetting is adequate to flush most of the cells from the bottom of the flasks. The morphological appearance of the cells is described in Schneider, J. Embryol. Exp. Morph. 27: 353-365 (1972). They are essentially epithelial-like in appearance and range from about 5-11μm in diameter and 11-35μm in length. Small pockets containing rounded cells may be dispersed randomly throughout the other cells.

Preferably, the Schneider 2 (S2) cells are maintained in Schneider's *Drosophila* medium plus 10% FBS including penicillin (100 unit/ml) and streptomycin (100mg/ml). It is preferable to keep the cells at a density of more than 0.5 X 10^5/ml, and to grow them at a 24-30 °C temperature range. The cells tend to double in fewer than 24 hours and grow to high cell density, i.e., about 2 X 10^7/ml or greater. The cells may also be frozen in 90% FBS and 10% DMSO, for later use or analysis. One may place the cells at -70 °C and then store in liquid nitrogen.

A preferred cell line according to the present invention, identified as Schneider 2 (S2) cells, has been deposited pursuant to Budapest Treaty requirements with the American Type Culture Collection (ATCC), Rockville, MD, on February 18, 1992, and was assigned accession number CRL 10974.

Cells of the present invention are transfected with cDNAs encoding (human) MHC heavy chains, β-2 microglobulin and one or more assisting molecules, which have each been inserted into (i.e., operatively linked to) an expression vector. In a more preferred embodiment, the vector comprises *Drosophila* expression plasmid pRmHa-3, into which expressible nucleotide sequences encoding human Class I MHC heavy chains, human β-2 microglobulin or human assisting molecules have been inserted using techniques disclosed herein. Preferably, the cDNAs encoding MHC heavy chains, those encoding β-2 microglobulin and those encoding assisting molecules are operatively linked to separate expression plasmids and are cotransfected into the cultured cells. Alternatively, the cDNAs encoding MHC heavy chains, β-2 microglobulin and assisting molecules may be operatively linked to the same expression plasmid and cotransfected via that same plasmid. In another variation, cDNAs

encoding MHC heavy chains, β-2 microglobulin, assisting molecules, and a cytokine such as IL-2 are operatively linked to expression plasmids and are cotransfected into a cell line of the present invention. Selection of HLA genes, construction of appropriate vectors and primer selection are described in greater detail above.

Successfully transformed cells, i.e., cells that contain an expressible human nucleotide sequence according to the present invention, can be identified via well-known techniques. For example, cells resulting from the introduction of a cDNA or rDNA of the present invention can be cloned to produce monoclonal colonies. Cells from those colonies can be harvested, lysed, and their DNA content examined for the presence of the rDNA using a method such as that described by Southern, J. Mol. Biol. 98: 503 (1975). In addition to directly assaying for the presence of rDNA, successful transformation or transfection may be confirmed by well-known immunological methods when the rDNA is capable of directing the expression of a subject chimeric polypeptide. For example, cells successfully transformed with an expression vector may produce proteins displaying particular antigenic properties which are easily determined using the appropriate antibodies. In addition, successful transformation/transfection may be ascertained via the use of an additional vector bearing a marker sequence, such as neomycin resistance, as described hereinabove.

It is also preferable that the culture be stabile and capable of sustained growth at reduced temperatures. For example, it is preferred that the culture be maintained at about room temperature, e.g., about 24-27 °C. In other embodiments, the culture is maintained at higher temperatures, particularly during the process of activating $CD8^+$ cells. It is thus

preferred that a culture according to the present invention be capable of withstanding a temperature challenge of about 30 °C to about 37 °C. Addition of β-2 microglobulin to a culture stabilizes the Class I MHC to at least a 30 °C challenge; addition of β-2 microglobulin and peptides results in greater thermostability at higher temperatures, i.e., at 37 °C.

In order to prepare the culture for expression of empty -- or more preferably, peptide-loaded -- MHC molecules, the culture may first require stimulation, e.g., via $CuSO_4$ induction, for a predetermined period of time. After a suitable induction period -- e.g., about 12-48 hours, peptides may be added at a predetermined concentration (e.g., about 100μg/ml). Peptides may be prepared as discussed below. After a further incubation period -- e.g., for about 12 hours at 27 °C -- the culture is ready for use in the activation of $CD8^+$ cells. While this additional incubation period may be shortened or perhaps omitted, the culture tends to become increasingly stable to temperature challenge if it is allowed to incubate for a time prior to addition of resting or naive $CD8^+$ cells. For example, cultures according to the present invention to which peptide has been added are capable of expressing significant amounts of peptide-loaded Class I MHC molecules even when incubated for extended periods of time at 37 °C.

Nutrient media useful in the culturing of transformed host cells are well known in the art and can be obtained from numerous commercial sources. In embodiments wherein the host cell is mammalian, a "serum-free" medium is preferably used.

4. Human β-2 microglobulin and Assisting Molecules

In order to establish a cell line capable of producing therapeutically useful amounts of

surface-expressed human Class I MHC molecules, it is preferable to cotransfect a cell line of the present invention with a vector operably linked to a nucleotide sequence encoding β-2 microglobulin in order to effect appropriate levels of expression of human MHC molecules in the cell line. While the nucleotide sequence encoding mammalian β-2 microglobulin such as mouse β-2 microglobulin increases the stability of the human Class I MHC molecules expressed in the cell lines of the present invention, it is preferable to cotransfect the cell line with a vector operably linked to an expressible nucleotide sequence encoding a human β-2 microglobulin.

As discussed above, a preferred vector according to the present invention includes a nucleotide sequence encoding at least a portion of a mammalian β-2 microglobulin molecule operatively linked to the vector for expression. The gene for the assisting molecules can be linked to the same or another vector. It is also feasible to construct a vector including nucleotide sequences encoding both a Class I MHC heavy chain and a β-2 microglobulin.

The sequencing and primers used for the assisting molecules are discussed in more detail below. However, the protocols are similar.

A human β-2 microglobulin cDNA sequence has been published (see Suggs, et al., PNAS 78: 6613-17, 1981) and the sequence was used as a template for a polymerase chain reaction (PCR) using the following primers:

5' primer:

5' GCTTGGATCCAGATCTACCATGTCTCGCTCCGTGGCCTTAGCTGTGCT CGCGCTACTCTC 3'

(SEQ ID NO 15)

3' primer

5' GGATCCGGATGGTTACATGTCGCGATCCCACTTAAC 3'

(SEQ ID NO 16)

The primers are used in a standard PCR reaction (see above and references cited therein). The reaction products are extracted with phenol, purified using a Geneclean kit (Bio 101, San Diego, CA), digested with Bam HI and cloned into the Bam HI site of pBS (Stratagene, La Jolla, CA). After verification of the sequence, this Bam HI fragment is cloned into the Bam HI site of an appropriate expression vector. In the preferred embodiment, human β-2 microglobulin cDNA is synthesized and operably linked to expression vector pRmHa-3.

5. Peptides

Virtually all cellular proteins in addition to viral antigens are capable of being used to generate relevant peptide fragments that serve as potential Class I MHC ligand. In most mammalian cells, then, any particular MHC peptide complex would represent only a small proportion of the total MHC encoded molecules found on the cell surface. Therefore, in order to produce surface-expressed human Class I MHC molecules that have an increased capacity to specifically activate CD8$^+$ cells, it is preferable to isolate and load peptide fragments of appropriate size and antigenic characteristics onto Class I molecules.

The peptides of the present invention bind to Class I MHC molecules. The binding occurs under biological conditions which can be created *in vivo* as well as *in vitro*. The exact nature of the binding of the peptides need not be known for practice of the invention.

In a preferred embodiment, the peptides to be loaded onto the Class I MHC molecules are antigenic. It is also preferred that the peptides be of a uniform size, preferably 8-mers or 9-mers, and most preferably, 8-mers. It is also preferable that the peptides prepared for

loading onto the MHC molecules be of a single species; i.e., that all peptides loaded onto the MHC be identical in size and sequence. In this manner, it is possible to produce monoantigenic peptide-loaded MHC molecules.

Peptides may be presented to the cells via various means. Preferably, peptides are presented in a manner which allows them to enter an intracellular pool of peptides. For example, peptides may be presented via osmotic loading. Typically, peptides are added to the culture medium. The peptides may be added to the culture in the form of an intact polypeptide or protein which is subsequently degraded via cellular processes, e.g., via enzymatic degradation. Alternatively, the intact polypeptide or protein may be degraded via some other means such as chemical digestion (e.g. cyanogen bromide) or proteases (e.g. chymotrypsin) prior to its addition to the cell culture. In other embodiments, the peptides are presented in smaller segments which may or may not comprise epitopic amino acid sequences.

In a preferred embodiment, a sufficient amount of protein(s) or peptide(s) is added to the cell culture to allow the Class I MHC molecules to bind and subsequently present a large density of the peptide -- preferably, with the same kind of peptide attached to each MHC -- on the surface of human Class I MHC-expressing cells of the present invention. It is also preferred to allow the human Class I MHC heavy chains and human β-2 microglobulin to bind -- i.e., to form heterodimers -- prior to presenting peptide to the MHC molecules intracellularly.

In another embodiment of the invention, peptides are added to transfected cells of the present invention in order to enhance the thermostability of the MHC molecules expressed by the cells. As noted above, peptides are preferably added to the culture medium.

Antigenic peptides that bind to the Class I molecules serve to thermostabilize the MHC molecules and also increase the cell surface expression. Cultures with added peptides which bind to the MHC molecules are thus significantly less susceptible to temperature challenge than cultures without added peptide.

In one embodiment of the present invention, antigenic peptides are presented to the transformed/transfected cell line in various forms. For example, an entire protein or other antigenic polypeptide may be degraded chemically or enzymatically, for example, and added to the cell line in this form. For example, a protein of interest is degraded with chymotrypsin and the resultant mixture of peptide "fragments" is added to a transformed or transfected cell culture; these cells are then allowed to "choose" the appropriate peptides (which are often smaller peptides, preferably 8mers or 9mers) to load onto the Class I MHC molecules. Alternatively, an entire protein or polypeptide sequence may be cloned into an appropriate vector and inserted into a procaryotic cell, whereby the cell generates significant amounts of the antigenic polypeptide which are then harvested, purified, and digested into peptides which are then added to the transformed/transfected eukaryotic cell culture. The cells again would be allowed to "choose" the peptides to load onto the expressed MHC.

6. Isolation of Resting or Precursor $CD8^+$ cells

Resting (or naive or precursor) $CD8^+$ cells -- i.e., T-cells that have not been activated to target a specific antigen -- are preferably extracted from the patient prior to incubation of the $CD8^+$ cells with the transformed cultures of the present invention. It is also preferred that precursor $CD8^+$ cells be harvested from a patient prior to the initiation of other treatment

or therapy which may interfere with the CD8· cells' ability to be specifically activated. For example, if one is intending to treat an individual with a neoplasia or tumor, it is preferable to obtain a sample of cells and culture same prior to the initiation of chemotherapy or radiation treatment.

Methods of extracting and culturing lymphocytes are well known. For example, U.S. Patent No. 4,690,915 to Rosenberg describes a method of obtaining large numbers of lymphocytes via lymphocytopheresis. Appropriate culturing conditions used are for mammalian cells, which are typically carried out at 37 °C.

Various methods are also available for separating out and/or enriching cultures of precursor CD8· cells. Some examples of general methods for cell separation include indirect binding of cells to specifically-coated surfaces. In another example, human peripheral blood lymphocytes (PBL), which include CD8· cells, are isolated by Ficoll-Hypaque gradient centrifugation (Pharmacia, Piscataway, NJ). PBL lymphoblasts may be used immediately thereafter or may be stored in liquid nitrogen after freezing in FBS containing 10% DMSO (Sigma Chemical Co., St. Louis, MO), which conserves cell viability and lymphocyte functions.

Alternative methods of separating out and/or enriching cultures of precursor cells include both positive and negative selection procedures. For positive selection, after lymphocyte-enriched PBL populations are prepared from whole blood, sub-populations of CD8· lymphocytes are isolated therefrom by affinity-based separation techniques directed at the presence of the CD8 receptor antigen. These affinity-based techniques include flow microfluorimetry, including fluorescence-activated cell sorting (FACS), cell adhesion, and like methods. (See, e.g., Scher and Mage,

in Fundamental Immunology, W.E. Paul, ed., pp. 767-780, River Press, NY (1984).) Affinity methods may utilize anti-CD8 receptor antibodies as the source of affinity reagent. Alternatively, the natural ligand, or ligand analogs, of CD8 receptor may be used as the affinity reagent. Various anti-T-cell and anti-CD8 monoclonal antibodies for use in these methods are generally available from a variety of commercial sources, including the American Type Culture Collection (Rockville, MD) and Pharmingen (San Diego, CA).

Negative selection procedures are utilized to effect the removal of non-CD8 from the $CD8^+$ population. This technique results in the enrichment of $CD8^+$ cells from the T- and B-cell population of leucophoresed patients. Depending upon the antigen designation, different antibodies may be appropriate. (For a discussion and review of nomenclature, antigen designation, and assigned antibodies for human leucocytes, including T-cells, see Knapp, et al., Immunology Today 10: 253-258 (1989) and Janeway et al., Immunobiology, supra.) For example, monoclonal antibodies OKT4 (anti-CD4, ATCC No. CRL 8002) OKT 5 (ATCC Nos. CRL 8013 and 8016), OKT 8 (anti-CD8, ATCC No. CRL 8014), and OKT 9 (ATCC No. CRL 8021) are identified in the ATCC Catalogue of Cell Lines and Hybridomas (ATCC, Rockville, MD) as being reactive with human T lymphocytes, human T-cell subsets, and activated T-cells, respectively. Various other antibodies are available for identifying and isolating T-cell species.

In a further embodiment, $CD8^+$ cells can be isolated by combining both negative and positive selection procedures. (See, e.g. Cai and Sprent, J. Exp. Med. 179: 2005-2015 (1994)).

Preferably, the PBLs are then purified. For example, Ficoll gradients may be utilized for this

purpose. The purified PBLs would then be mixed with syngeneic *Drosophila* cells preincubated with the appropriate antigenic peptides.

7. *In Vitro* Activation of CD8⁺ Cells

In order to optimize the *in vitro* conditions for the generation of specific cytotoxic T-cells, the culture of antigen-presenting cells is maintained in an appropriate medium. In the preferred embodiment, the antigen-presenting cells are *Drosophila* cells, which are preferably maintained in serum-free medium (e.g. Excell 400).

Prior to incubation of the antigen-presenting cells with the cells to be activated, e.g., precursor CD8⁺ cells, an amount of antigenic peptide is added to the antigen-presenting cell culture, of sufficient quantity to become loaded onto the human Class I molecules to be expressed on the surface of the antigen-presenting cells. According to the present invention, a sufficient amount of peptide is an amount that will allow about 200 to about 500,000 and preferably about 200 to 1,000 or more, human Class I MHC molecules loaded with peptide to be expressed on the surface of each antigen-presenting cell. Preferably, the antigen-presenting cells are incubated with >20μg/ml peptide.

Resting or precursor CD8⁺ cells are then incubated in culture with the appropriate antigen-presenting cells for a time period sufficient to activate and further enrich for a population of CD8⁺ cells. Preferably, the CD8⁺ cells shall thus be activated in an antigen-specific manner. The ratio of resting or precursor CD8⁺ (effector) cells to antigen-presenting cells may vary from individual to individual and may further depend upon variables such as the amenability of an individual's lymphocytes to

culturing conditions and the nature and severity of the disease condition or other condition for which the within-described treatment modality is used. Preferably, however, the lymphocyte:antigen-presenting cell (e.g. *Drosophila* cell) ratio is preferably in the range of about 30:1 to 300:1. For example, in one embodiment, 3 X 10^7 human PBL and 1 X 10^6 live *Drosophila* cells were admixed and maintained in 20 ml of RPMI 1640 culture medium.

The effector/antigen-presenting culture may be maintained for as long a time as is necessary to activate and enrich for a population of a therapeutically useable or effective number of $CD8^+$ cells. In general terms, the optimum time is between about one and five days, with a "plateau" -- i.e. a "maximum" specific $CD8^+$ activation level -- generally being observed after five days of culture. In one embodiment of the present invention, *in vitro* activation of $CD8^+$ cells is detected within a brief period of time after transfection of a cell line. In one embodiment, transient expression in a transfected cell line capable of activating $CD8^+$ cells is detectable within 48 hours of transfection. This clearly indicates that either stable or transient cultures of transformed cells expressing human Class I MHC molecules are effective in activating $CD8^+$ cells.

Preferably, the enrichment and concordant activation of $CD8^+$ cells is optimal within one week of exposure to antigen-presenting cells. Thereafter, in a preferred embodiment, the enriched and activated $CD8^+$ cells are further purified by isolation procedures including site restriction, rosetting with antibody-red blood cell preparations, column chromatography and the like. Following the purification, the resulting $CD8^+$ cell preparation is further expanded by maintenance in culture for a period of time to obtain a population of

10^7 activated $CD8^+$ cells. This period may vary depending on the replication time of the cells but may generally be 14 days. Activation and expansion of $CD8^+$ cells has been described by Riddell et al., Curr. Opin. Immunol., 5: 484-491 (1993).

8. Separation of $CD8^+$ Cells from *Drosophila* Cells

Activated $CD8^+$ cells may be effectively separated from the stimulator (e.g., *Drosophila*) cells using one of a variety of known methods. For example, monoclonal antibodies specific for the stimulator cells, for the peptides loaded onto the stimulator cells, or for the $CD8^+$ cells (or a segment thereof) may be utilized to bind their appropriate complementary ligand. Antibody-tagged cells may then be extracted from the stimulator-effector cell admixture via appropriate means, e.g., via well-known immunoprecipitation or immunoassay methods.

9. Administration of Activated $CD8^+$ Cells

Effective, cytotoxic amounts of the activated $CD8^+$ cells can vary between *in vitro* and *in vivo* uses, as well as with the amount and type of cells that are the ultimate target of these killer cells. The amount will also vary depending on the condition of the patient and should be determined via consideration of all appropriate factors by the practitioner. Preferably, however, about 1×10^6 to about 1×10^{12}, more preferably about 1×10^8 to about 1×10^{11}, and even more preferably, about 1×10^9 to about 1×10^{10} activated $CD8^+$ cells are utilized for adult humans, compared to about $5 \times 10^6 - 5 \times 10^7$ cells used in mice.

Preferably, as discussed above, the activated $CD8^+$ cells are harvested from the *Drosophila* cell culture prior to administration of the $CD8^+$ cells to the individual being treated. It is important to note,

however, that unlike other present and proposed treatment modalities, the present method uses a cell culture system (i.e., *Drosophila* cells) that are not tumorigenic. Therefore, if complete separation of *Drosophila* cells and activated $CD8^+$ cells is not achieved, there is no inherent danger known to be associated with the administration of a small number of *Drosophila* cells, whereas administration of mammalian tumor-promoting cells may be extremely hazardous.

Methods of re-introducing cellular components are known in the art and include procedures such as those exemplified in U.S. Patent No. 4,844,893 to Honsik, et al. and U.S. Patent No. 4,690,915 to Rosenberg. For example, administration of activated $CD8^+$ cells via intravenous infusion is appropriate.

10. HLA Typing

As noted previously, HLA haplotypes/allotypes vary from individual to individual and, while it is not essential to the practice of the present invention, it is often helpful to determine the individual's HLA type. The HLA type may be determined via standard typing procedures and the PBLs purified by Ficoll gradients. The purified PBLs would then be mixed with syngeneic *Drosophila* cells preincubated with the appropriate antigenic peptides -- e.g., in therapeutic applications relating to viral infections, cancers, or malignancies, peptides derived from viral- or cancer-specific proteins.

Continuing to use viral or malignant conditions as an example, in those instances in which specific peptides of a particular viral- or cancer-specific antigen have been characterized, the synthesized peptides encoding these epitopes will preferably be used. In cases in which the preferred antigenic peptides have not been precisely determined, protease digests of viral- or cancer-specific proteins may be used. As a source for

such antigen, cDNA encoding viral- or cancer-specific proteins is cloned into a bacterial expression plasmid and used to transform bacteria, e.g., via methods disclosed herein.

After HLA typing, if *Drosophila* cells expressing the preferred HLA are not available, cDNAs encoding the preferred HLA may be cloned via use of the polymerase chain reaction. The primers disclosed in section B.1. above (SEQ ID NO 1 through SEQ ID NO 12) may be used to amplify the appropriate HLA-A, -B, -C, -E, -F, or -G cDNAs in separate reactions which may then be cloned and sequenced as described in the methods disclosed for HLA A2.1 below. Stable cell lines expressing the cloned HLA may then be established in the *Drosophila* cells. Alternatively, a population of insect cells transiently expressing a bulk population of cloned recombinant molecules from the PCR reaction may be used for *in vitro* CD8$^+$ activation.

EXAMPLES

The following examples are intended to illustrate, but not limit, the present invention.

Example 1

Expression of Human Class I MHC Molecules

A. Preparation of pRmHa-3 Expression Vector

The pRmHa-3 expression vector for use in expressing MHC proteins in *Drosophila* Schneider 2 (S2) cells as described in this invention was constructed by ligating a Sph I linearized pRmHa-1 DNA expression vector with a DNA fragment resulting from a Sph I restriction digest of a pRmHa-2 expression vector as described below. The ligating of pRmHa-1 with the pRmHa-2 fragment in this manner was performed to remove one of two Eco RI restriction endonuclease cloning sites present in pRmHa-1. Thus, the resultant pRmHa-3 expression vector contained only one Eco RI restriction site in the

multiple cloning site (polylinker) into which various MHC-encoding DNA fragments were inserted as described in the Examples.

1. Preparation of pRmHa-1 Expression Vector

The pRmHa-1 expression vector, containing a metallothionein promoter, metal response consensus sequences (designated MT) and an alcohol dehydrogenase (ADH) gene containing a polyadenylation signal isolated from *Drosophila melanogaster*, was constructed as described by Bunch et al., Nucl. Acids Res. 16: 1043-61 (1988). A schematic of the final pRmHa-1 construct is shown in Figure 2. The plasmid expression vector, pUC18, having the ATCC accession number 37253, was used as the source vector from which subsequent vectors described herein were derived. The pUC18 plasmid contains the following restriction sites from 5' to 3' in the multiple cloning site, all of which are not illustrated in the schematic representations of the pUC18-derived vectors in Figure 1: Eco RI; Sac I; Kpn I; Sma I and Sma I located at the same position; Bam HI; Xba I; Sal I, Acc I and Hinc II located at the same position; Pst I; Sph I and Hind III. The pUC18 vector was first digested with Hind III to form a linearized pUC18. Blunt ends were then created by filling in the Hind III ends with DNA polymerase I large fragment as described by Maniatis et al., Molecular Cloning: A Laboratory Manual, eds. Cold Spring Harbor Laboratory, New York (1982).

The resultant linearized blunt-ended pUC18 vector was ligated with a 740 base pair (bp) Hinf I fragment from the *Drosophila melanogaster* ADH gene containing a polyadenylation signal. The ligated ADH allele was first isolated from the plasmid pSACI, described by Goldberg et al., PNAS USA 77: 5794-5798 (1980), by digestion with Hinf I followed by blunt ending with Klenow resulting in the nucleotide sequence listed

in SEQ ID NO 14. The pSACI vector containing the ADH allele was constructed by subcloning into pBR322 (ATCC accession number 31344) a 4.7 kilobase (kb) Eco RI fragment of *Drosophila* DNA selected from a bacteriophage lambda library containing random, high molecular weight (greater than 15 kb). The 5' Hinf I restriction site occurred naturally in the ADH gene at position 1770 as described by Kreitman, Nature 304: 412-417 (1983). The 3' Hinf I site was derived from the pUC18 vector into which the ADH gene had been cloned. This position was four bases 3' to the Xba I site at position 2500 of the ADH gene. The ADH segment extended from the 35 bp upstream of the polyadenylation/cleavage sequence in the 3' untranslated portion of the ADH mRNA to 700 bp downstream of the polyadenylation signal. The resultant pUC18-derived vector containing the ADH gene fragment was designated pHA-1 as shown in Figure 1.

The 421 bp Eco RI/Stu I MT gene fragment was obtained from a clone containing DNA of approximately 15.3 kb in a *Drosophila melanogaster* genomic DNA library. The library, prepared with a Mbo I partial digestion of imaginal DNA, was cloned in the lambda derivative EMBL4. The fragment contained the MT promoter and metal response consensus elements of the *Drosophila* MT gene (Maroni et al., Genetics 112: 493-504 (1986)). This region, containing the promoter and transcription start site at nucleotide 1+, corresponded to position -370 to nucleotide position +54 of the MT gene (SEQ ID NO 13). The resultant fragment was then ligated into pHA-1 expression vector prepared above that was previously linearized with Eco RI and Sma I. The 3' blunt end in MT created by the Stu I digest was compatible with the blunt end in pHA-1 created by the Sma I digest. The resultant pUC18-derived vector containing a 5' Drosophila MT gene fragment and a 3' ADH gene fragment was designated

pRmHa-1. The pRmHa-1 expression vector, shown in Figure 2, contained the origin of replication (ori) and the beta-lactamase gene conferring resistance to ampicillin (Amp') from pUC18 as shown in Figure 1 on the pHa-1 vector. The diagram of pRmHa-1 also shows the 5' to 3' contiguous positions of the MT gene fragment, the multiple cloning site and the ADH gene fragment. The pRmHa-1 vector was used as described in c. below in the construction of the pRmHa-3 expression vector.

2. Preparation of pRmHa-2 Expression Vector

The construction of pRmHa-2 is shown in Figure 1. For constructing the pRmHa-2 expression vector, the MT fragment prepared above was inserted into the pUC18-derived vector pHA-1 as described for constructing pRmHa-1 above with a few modifications. An Eco RI linker was added to the Stu I site of the Eco RI/Stu I-isolated MT gene fragment prepared above to form a metallothionein fragment having Eco RI restriction sites on both ends. The resultant fragment was then ligated into the ADH fragment-containing pUC18 expression vector that was previously linearized with Eco RI. The resultant pUC18-derived vector containing a 5' Drosophila MT gene fragment and a 3' ADH gene fragment having two Eco RI restriction sites 5' to the multiple cloning site was designated pRmHa-2. The pRmHa-2 expression vector, shown in Figure 1, contained the origin of replication (ori) and the beta-lactamase gene conferring resistance to ampicillin (Amp') from pUC18. The diagram of pRmHa-2 also shows the 5' to 3' contiguous positions of the MT gene fragment, the multiple cloning site and the ADH gene fragment. The pRmHa-2 vector was used along with pRmHa-1 as described in c. below in the construction of the pRmHa-3 expression vector.

3. Preparation of pRmHa-3 Expression Vector

To prepare the pRmHa-3 expression vector that had only one Eco RI restriction site, a fragment from pRmHa-2 was ligated into pRmHa-1. For this construction, pRmHa-2, prepared in b. above, was first digested with Sph I. The resultant Sph I fragment beginning in the middle of the MT gene and extending to the Sph I site in the multiple cloning site was first isolated from the pRmHa-2 vector and then ligated into pRmHa-1 prepared in A.1. above. The pRmHa-1 vector was previously modified to remove the Eco RI restriction site 5' to the MT gene fragment then linearized with Sph I. This process is schematically illustrated in Figure 2. To remove the Eco RI site in pRmHa-1, the vector was first digested with Eco RI to form a linearized vector, then blunt ended with Mung Bean nuclease and religated.

The pRmHa-1 vector lacking an Eco RI site was then digested with Sph I to remove the region corresponding to the Sph I fragment insert from pRmHa-2 and form a linearized pRmHa-1 vector. The Sph I fragment from pRmHa-2 was then ligated into the Sph I linearized pRmHa-1 to form the pRmHa-3 expression vector. A schematic of the pRmHa-3 vector is shown in Figure 3. The relative positions of the various restriction sites from the pUC18 vector from which pRmHa-3 was derived are indicated on the figure. In addition, the relative positions and lengths of the MT and ADH gene fragments separated by the multiple cloning site (polylinker) into which the MHC gene of interest is cloned are indicated on the figure. The pRmHa-3 vector, being derived from pUC18, contains the pUC18 origin of replication and beta-lactamase gene conferring ampicillin resistance. Thus, MHC encoding DNA fragments as prepared in this invention and cloned into the multiple cloning site of

pRmHa-3 were transcriptionally regulated by the MT promoter and polyadenylated via the ADH gene.

B. cDNA Synthesis

Detailed descriptions of the cDNA of Class I MHC molecules of various HLA groups can be found in U.S. Patent No. 5,314,813 to Peterson et al. which has been incorporated by reference.

cDNAs encoding any preferred HLA may be cloned via use of the polymerase chain reaction. The primers disclosed in section B.1. above (SEQ ID NO 1 through SEQ ID NO 12) may be used to amplify the appropriate HLA-A, -B, -C, -E, -F, or -G cDNAs in separate reactions which may then be cloned and sequenced as described in the methods disclosed for HLA A2.1 above. Preparation of cDNA from human cells is carried out as described in Ennis, et al., PNAS USA 87: 2833-2837 (1990). Briefly, a blood sample is obtained from the individual and cells are collected after centrifugation and used to prepare total RNA. First strand cDNA is synthesized by using oligo(dT) and avian myeloblastosis virus reverse transcriptase. The resulting cDNA is used in a PCR amplification reaction utilizing the appropriate primer(s) as noted in section B.1. above, and a GeneAmp kit and thermal cycler (Perkin-Elmer/Cetus). Reaction conditions are preferably as follows. 100ng cDNA template and 50 picomoles of each oligonucleotide primer are used. Thirty cycles are run as follows: (a) one minute at 94 °C; (b) one minute at 60 °C; and (c) one minute, 30 seconds at 72 °C. The PCR reaction is then heated to 100 °C for 10 minutes to kill the Taq polymerase and the ends of the DNA made blunt by T4 polymerase (Stratagene, San Diego, CA).

To synthesize HLA A2.2, cDNA encoding a complete A2.2 (see Holmes, et al., J. Immunol. 139: 936-41 (1987), for the published sequence) is cloned into

an M13mp19 plasmid, a commercially available bacteriophage vector (Stratagene, La Jolla, CA). cDNA is synthesized by PCR using primers derived from the published sequence of A2. The cDNA is released from an M13mp19 clone as a Not I (overhang filled with Klenow)/Eco RI fragment. (Klenow fragments are part of the *E. coli* DNA polymerase I molecule, produced by the treatment of *E. coli* DNA pol I with subtilisin. They are used to "fill out" 5' or 3' overhangs at the ends of DNA molecules produced by restriction nucleases.) The Not I/Eco RI fragment is inserted into pSP64T digested with Bg III (ends filled with Klenow) and Eco RI. pSP64T is an SP6 cloning vector designed to provide 5' and 3' flanking regions from an mRNA which is efficiently translated (β-globin) to any cDNA which contains its own initiation codon. This translation SP6 vector was constructed by digesting pSP64-Xβm with Bal I and Bst EII, filling in the staggered ends with T4 DNA polymerase and adding a Bgl II linker by ligation. Bal I cuts the β-globin cDNA two bases upstream of the ATG (start codon) and Bst EII cuts eight bases upstream of the TAA (stop codon). There is only one Bgl II site in pSP64T so that restriction enzymes cutting in the polylinker fragment, from Pst I to Eco RI can still be used to linearize the plasmid for transcription. (See Kreig and Melton, Nucleic Acid Res. 12: 7057-7070, (1984), which also describes the construction of the plasmid pSP64-Xβm.) The resulting plasmid is cleaved with Eco RI (end filled with Klenow) and Hind III which is cloned into the pCMUII polylinker between Hind III (5') and Stu I (3'). (See Paabo, et al., EMBO J. 5: 1921-1927 (1986).) The entire cDNA is removed as a Hind III (end filled with Klenow) Bam HI fragment which is cloned into pRmHa-3 cleaved with Sma I and Bam HI.

HLA A2.2 soluble form was prepared by engineering a stop codon into the above-described A2.2 cDNA immediately preceding the transmembrane domain. The modification is achieved by cleaving the A2.2 cDNA cloned in the eukaryotic expression vector pCMUII between Hind III 5' and Stu I 3' (see above) with Mbo II and Bam HI inserting the following oligonucleotides:

5' primer: 5' GGAGCCGTGACTGACTGAG 3' (SEQ ID NO 17)

3' primer: 5' CCCTCGGCACTGACTGACTCCTAG 3' (SEQ ID NO 18)

The resulting recombinant plasmid is cleaved with Hind III, the overhanging end filled with Klenow, then cut with Bam HI releasing a restriction fragment which is cloned into pRmHa-3 in the same way as A2.2 full length.

1. Construction of Murine ICAM-1 Expression Vector

Spleen cells were isolated from Balb/c mice. The spleen cells were stimulated with conA; mRNA was isolated using the FastTrack kit (Invitrogen, San Diego, CA) according to the manufacturers' instructions. cDNA was synthesized from the mRNA using AMV reverse transcriptase kit (Promega, Madison, WI) according to the manufacturers' instructions. Based on the published cDNA nucleotide sequence (Siu, G. et al., J. Immunol. 143, 3813-3820 (1989) the following oligonucleotides were synthesized as PCR primers:

5': TTTAGAATTCAC CATGGCTTCA ACCCGTGCCA AG

3': TTTAGTCGACTC AGGGAGGTGG GGCTTGTCC

The cDNA synthesized was subjected to PCR using these primers. The product was cleaved with the restriction enzymes Eco RI and Sal I and ligated into pRmHa-3, which had been digested with the restriction enzymes Eco RI and Sal I.

2. Construction of Murine B7.1 Expression Vector

Spleen calls were isolated from Balb/c mice and stimulated with conA. Messenger RNA was isolated using the FastTrack kit (Invitrogen, San Diego, CA) according to the manufacturer's instructions. cDNA was synthesized from the mRNA using AMV reverse transcriptase kit (Promega, Madison, WI) according to the manufacturer's instructions.

Based on the published cDNA nucleotide sequence (Freeman, et al., J. Exp. Med. 174: 625-631 (1991)) the following oligonucleotides were synthesized as PCR primers:

5': TTTAGAATTCAC CATGGCTTGC AATTGTCAGT TG

3': TTTAGTCGACCT AAAGGAAGAC GGTCTGTTC

The cDNA synthesized was subjected to PCR using these primers. The product was cleaved with the restriction enzymes Eco RI and Sal I and ligated into pRmHa-3, which had been digested with the restriction enzymes Eco RI and Sal I.

3. Construction of Murine B7.2 Expression Vector

IC-21 cells (obtained from ATCC) were propagated in RPMI 1640 medium containing 10% Fetal Calf Serum. mRNA was isolated from these cells using the FastTrack kit (Invitrogen, San Diego, CA) according to the manufacturer's instructions. cDNA was synthesized from the mRNA using AMV reverse transcriptase kit (Promega, Madison., WI) according to the manufacturer's instructions. Based on the published cDNA nucleotide sequence (Freeman, et al., J. Exp. Med. 178: 2185-2192 (1993)) the following oligonucleotides were synthesized as PCR primers:

5': TTTAGAATTCAC CATGGACCCC AGATGCACCA TGGG

3': TTTAGTCGACTC ACTCTGCATT TGGTTTTGCT GA

The cDNA synthesized was subjected to PCR using these primers. The product was cleaved with the restriction

enzymes Eco RI and Sal I and ligated into pRmHa-3, which had been digested with the restriction enzymes Eco RI and Sal I.

The above expression constructs were transfected into *Drosophila* S2 cells using the calcium phosphate method as listed in Table 1. Stable cell lines were selected by including 500 μg/ml Geneticin in the cell culture medium.

TABLE 1

Transfected Cells		MHC I (L^d) μg	β2 μg	B7.1 (CD 80) μg	B7.2 μg	ICAM-1 (CD54) μg	phsneo μg
1	A	12	12				1
2	B	8	8	8			1
3	C	8	8		8		1
4	C	8	8			8	1
5	D	6	6	6		6	1
6	E	6	6		6	6	1
7	F	6	6	6	6		1
8	G	4.8	4.8	4.8	4.8	4.8	1

Human accessory and costimulatory molecules were cloned from human cell lines demonstrated to express these proteins by FACS analysis with monoclonal antibodies specific for the particular proteins. Adhesion molecules belonging to the integrin family, ICAM-I (CD54) and LFA-3 (CD58), were cloned from human cell lines K562 and HL60, respectively. The K562 cells, originated from human chronic myelogenous leukemia, were obtained from ATCC (CCL-243) and cultured under conditions recommended (i.e., RPMI with 10% fetal calf serum at 37 degrees C with 5% CO_2). HL60 cells, originated from a human promyelocytic leukemia, and were obtained from ATCC (CCL-240) and were cultured according

to ATCC's recommendations. Costimulatory molecules B7.1 and B7.2 were also cloned from K562 and HL60 cells respectively.

4. cDNA

Messenger RNA samples were prepared from each cell line from RNA isolated by the modified guanidinium thiocyanate method (Chromczynski, et al. Anal. Biochem. 162: 156-159, 1987) followed by poly A+ RNA selection on oligo(dt)-cellulose columns (Sambrook, J., et al, Molecular Cloning: A Laboratory Manual, Second Edition, 6.22-6.34, Cold Spring Harbor laboratory, CSH, NY), Induction of HL60 cells with vitamin D3 (usually required to express some cell surface molecules) was not required to obtain the B7.2 and LFA-3 molecules, the proteins were expressed in the absence of induction. cDNA was prepared using AMV reverse transcriptase kit according to the manufacturers' instructions (Promega, Madison WI).

5. PCR Primers

PCR primers were designed and synthesized after obtaining copies of the known sequences from the GENEWORKS database (Intelligenetics) and considering the ends needed to clone into the appropriate vectors. They are as follows with the top sequence of each protein the 5'primer and the bottom one the 3'primer:

B7.1 5'-ACCCTTGAAT CCATGGGCCA CACACGGAGG CAG-3'
5'-ATTACCGGAT CCTTATACAG GGCGTACACT TTCCCTTCT-3'

B7.2 5'-ACCCTTGAGC TCATGGATCC CCAGTGCACT ATG-3'
5'-ATTACCCCCG GGTTAAAAAC ATGTATCACT TTTGTCGCAT GA-3'

LFA-3 5'-ACCCTTGAGC TCATGGTTGC TGGGAGCGAC GCGGGG-3'
3'-ATTACCGGAT CCTTAAAGAA CATTCATATA CAGCACAATA CA-3'

ICAM-1 5'-ACCCTTGAAT TCATGGCTCC CAGCAGCCCC CGGCCC-3'
3'-ATTACCGGAT CCTCAGGGAG GCGTGGCTTG TGTGTTCGG-3'

6. Expression of DNA Fragment

The cDNA preparations from each of the cell lines was used to clone the desired proteins. The polymerase chain reaction was used to generate cDNA fragments utilizing the appropriate PCR primer (see above). The appropriate DNA fragments were cloned into the Drosophila fly vector pRMHA-3. Plasmid preparations have been prepared from all of the preparations and are now ready for transfection into the fly cells.

Human β-2 microglobulin cDNA is prepared using a published partial cDNA sequence (see Suggs, et al., PNAS 78: 6613-17, 1981) is used as a template for a polymerase chain reaction (PCR) with the following primers:

5' primer

5' GCTTGGATCCAGATCTACCATGTCTCGCTCCGTGGCCTTAGCTGTGCTCGC GCTACTCTC 3'

(SEQ ID NO 15)

3' primer

5' GGATCCGGATGGTTACATGTCGCGATCCCACTTAAC 3'

(SEQ ID NO 16)

The primers are used in a standard PCR reaction (see Nilsson, et al., Cell 58: 707 (1989)). The reaction products are extracted with phenol, purified using a Geneclean kit (Bio 101, San Diego, CA), digested with Bam HI and cloned into the Bam HI site of pBS (Stratagene, La Jolla, CA). After verification of the sequence, this Bam HI fragment is cloned into the Bam HI site of pRmHa-3.

As noted in the Examples, murine Class I cDNA was utilized in various instances. Murine Class I cDNA was prepared as follows.

H-2K^b: cDNA encoding a complete K^b molecule is obtained from an expression plasmid pCMU/K^b constructed as follows. A partial H-2K^b cDNA missing the leader

sequence and most of the alpha I domain is prepared according to the method of Reyes, et al., PNAS 79: 3270-74 (1982), producing pH202. This cDNA is used to generate a full-length molecule. The missing sequence is provided using a genomic clone encoding H-2K^b (Caligan, et al., Nature 291: 35-39, 1981) as a template in a PCR reaction, using a 5' primer flanked by a Not I site, followed by 21 nucleotides encoding the last seven amino acids of the leader sequence and 18 nucleotides complementary to the beginning of the alpha I domain and a 3' primer complementary to the region encompassing the Sty I site. The resulting fragment is ligated with pH202 at the Sty I site. The 5' sequence encoding the remainder of the signal sequence is obtained form the D^b cDNA (see below) as a Bam HI/Not I fragment. The entire coding sequence is cleaved from the expression plasmid as a Bam HI fragment and cloned into pRmHa-3 cleaved with Bam HI.

H-2L^d: cDNA encoding a complete L^d molecule is obtained from an expression plasmid pCMUIV/L^d (see Joly and Oldstone, Gene 97: 213, 1991). The complete cDNA is cleaved from a eukaryotic expression vector pCMU IV/L^d as a Bam HI fragment and cloned into pRmHa-3 as K^b.

As noted previously, the pCMU vector (pCMUIV) is derived from eukaryotic expression vector pC81G as described in Nilsson, et al., *supra*. Vector pC81G, in turn, is derived from pA81G (Paabo, et al., Cell 33: 445-453 (1983)) according to the method disclosed in Paabo, et al., EMBO J. 5: 1921-7 (1986).

H-2D^b: cDNA encoding a complete D^b molecule is obtained from expression plasmid pCMUIV/D^b (see Joly and Oldstone, Science 253: 1283-85, 1991). The complete cDNA is cleaved from a eukaryotic expression vector pCMUIV/D^b as a Bam HI fragment and cloned into pRmHa-3 as K^b.

Murine β-2 microglobulin: full-length murine β-2 microglobulin cDNA is obtained as a Hind III (5') (filled with Klenow)/Bgl II (3') fragment from pSV2neo (ATCC No. 37149) mouse β-2 microglobulin cDNA and cloned into pRmHa-3 cleaved with Sma I and Bam HI.

Vector phshsneo confers neomycin (G418) resistance and is a derivative of phsneo (pUChsneo) with an additional heat-shock promoter (hs) sequence, which may be synthesized from commercially-available pUC8 as described in Steller, et al., EMBO J. 4: 167 (1985). The heat shock promoter contained in these vectors is the *hsp*70 promoter. Other useful vectors conferring neomycin resistance (G418 resistance) include cosmid vector smart2 (ATCC 37588), which is expressed under the control of *Drosophila hsp*70 promoter, and plasmid vector pcopneo (ATCC 37409).

C. Insertion of Genes into Expression Vectors

The restriction products are subjected to electrophoresis on a 1% agarose gel (Maniatis, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory (1982)). The restriction fragments encoding the cDNAs are excised from the gel and purified away from the agarose using "Geneclean", according to manufacturers' directions (Bio 101, San Diego, CA). The expression plasmid pRmHa-3 (Figure 3) is cleaved with the appropriate restriction enzymes in One Phor All buffer according to the manufacturer's directions (Pharmacia, Piscataway, NJ) and treated with alkaline phosphatase as described in the manufacturer's literature (Boehringer Mannheim, Indianapolis, IN). One hundred ng of cleaved and phosphatased pRmHa-3 vector is mixed with 300ng of agarose gel purified Class I MHC heavy chain cDNA or β-2 microglobulin cDNA and ligated using T4 DNA ligase and One Phor all buffer as described in the manufacturers' literature. After incubation at 16 °C for five hours,

the ligation mixture is used to transform competent *E. coli* JM83 (Maniatis, et al., *supra* (1982)).

Methods disclosed in Maniatis, et al., *supra* are used to prepare the cDNA needed. The presence of the MHC heavy chain cDNA and its orientation in the vector is determined by restriction mapping. Bacteria containing the vector with the cDNA in the correct orientation relative to the metallothionein promoter are used for large scale preparation of DNA using the alkaline lysis method and cesium chloride gradient purification. The amount of DNA obtained is determined spectrophotometrically.

D. Transfection and Labeling of S2 Cells

S2 cells are grown in Schneider medium (Gibco/BRL, Grand Island, NY) supplemented with 10% fetal calf serum (heat treated for one hour at 55 °C), 100 units/ml penicillin, 100mg/ml streptomycin, and 1mM glutamine. (For convenience, this supplemented medium is hereinafter referred to as Schneider medium.) Cells are grown at 27 °C and typically passaged every seven days by diluting 1:17 in fresh medium. Cells are converted to growth in serum free media (Excell 400 or 401 supplemented with 100 units/ml penicillin, 100 mg/ml streptomycin, 1mM glutamine, and 500μg/ml G418 (JRH Biosciences, Lenexa, KS) by initial dilution at 50% Schneider/50% Excell 401. One week later, cells may be passaged into 10% Schneider medium/90% Excell 401 and one week later into 100% Excell 401. Cells are maintained in this medium and passaged every seven days by diluting 2:17 in fresh medium.

15 X 10^7 S2 cells at a concentration of 10^6 cells per ml are plated out in 85mm petri dishes. Twelve hours later, calcium phosphate/DNA precipitates, prepared as described below (1 ml) are added dropwise to the cells. After 48 hours, the supernatant is carefully

removed and the cells transferred to a 175cm^2 flask in a total volume of 50 ml in Schneider medium containing 500μg/ml Geneticin (G418) (Gibco/BRL, Grand Island, NY). After 21 days, 20 ml of the culture is removed to a fresh flask containing 30 ml of Schneider medium containing 500μg/ml G418. Ten days later, a stable population of cells that weakly adhered to the flask and grew with a doubling time of approximately 24 hours is obtained and these cells are subsequently cultured and passaged in the selection media as described above. Frozen aliquots of these cells are prepared by collecting 5-20 X 10^6 cells by centrifugation and resuspending them in 1 ml of cell freezing media (93% fetal calf serum/7% dimethylsulfoxide). Aliquots are then placed at -70 °C for one week and subsequently transferred to liquid nitrogen storage.

Calcium phosphate precipitates are prepared as described by Paabo, et al. (EMBO J. 5: 1921-27 (1986)), except that 25μg of DNA is used per transfection. The following combinations of DNA are used to prepare the indicated transfectant:

(a) MHC Class I heavy chain alone: 23μg heavy chain expression vector DNA + 2μg of phshsneo DNA.

(b) MHC Class I heavy chain + β-2 microglobulin: 11.5μg heavy chain expression vector DNA + 11.5μg of β-2 microglobulin (human or mouse) expression vector DNA + 2μg of phshsneo DNA.

Other combinations of mouse genes are presented in Table 1.

Twenty-four hours prior to metabolic labeling, cells are plated out at a cell density of 3-5 X $10^{}$ cells/ml (10 ml/85mm petri dish) in Schneider medium containing 1mM $CuSO_4$. Thirty minutes prior to labelling the medium is aspirated from the dishes and the cells are washed with 2 X 10 ml of PBS and then incubated in Graces

insect medium minus methionine and cysteine (special order from Gibco/BRL, Grand Island, NY) for 20 minutes, and then in 1 ml of this medium containing 0.1mCi ^{35}S Trans label (New England Nuclear; duPont, Boston, MA). After the labelling period, the labelling solution is aspirated and the cells are either lysed immediately on ice, with ice cold PBS/1% Triton X100 (1 ml) or after a chase period in the presence of methionine containing Schneider or Excell 400 medium (5 ml) (JRH Biosciences). The chase medium is collected if soluble Class I MHC molecules are being analyzed.

The following operations are all carried out with the lysates kept cold (less than 8 °C). The lysates were collected into Eppendorf tubes, centrifuged in a microfuge tube for 15 minutes at 13,000 X g, transferred to a fresh tube containing 100μl of a 10% slurry of protein A sepharose and placed on an end-over-end rotator for two hours. Following a further centrifugation in the microfuge for 15 minutes, the cell lysates are ready for analysis.

In experiments utilizing murine MHC, S2 cells were transfected with the murine MHC recombinants described above using the $CaPO_4$ precipitation method; each heavy chain is transfected either alone or as a 50:50 mix with the vector encoding β-2 microglobulin. A plasmid encoding neomycin resistance, phshsneo DNA, is included in each transfection such that a population of cells that stably expressed MHC Class I could be obtained by growing the transfectants in selection medium (Geneticin G418-sulphate, Gibco/BRL, Grand Island, NY).

E. Peptide Generation

Antigenic peptides according to the present invention may be obtained from naturally-occurring sources or may be synthesized using known methods. In various examples disclosed herein, peptides are

synthesized on an Applied Biosystems synthesizer, ABI 431A (Foster City, CA) and subsequently purified by HPLC.

Isolation or synthesis of "random" peptides may also be appropriate, particularly when one is attempting to ascertain a particular epitope in order to load an empty MHC molecule with a peptide most likely to stimulate precursor $CD8^+$ cells. One may produce a mixture of "random" peptides via use of proteasomes (see, e.g., Example 2.B.6) or by subjecting a protein or polypeptide to a degradative process -- e.g., digestion with chymotrypsin -- or peptides may be synthesized. While we have observed that the cell lines of the present invention are able to degrade proteins and polypeptides into smaller peptides capable of being loaded onto human Class I MHC molecules, it is preferable to introduce smaller peptides -- e.g., 8-mers and 9-mers -- directly into the cell culture to facilitate a more rapid loading and expression process.

If one is synthesizing peptides, e.g., random 8-, 9- and 18-amino acid peptides, all varieties of amino acids are preferably incorporated during each cycle of the synthesis. It should be noted, however, that various parameters --e.g., solvent incompatibility of certain amino acids -- may result in a mixture which contains peptides lacking certain amino acids. The process should thus be adjusted as needed -- i.e., by altering solvents and reaction conditions -- to produce the greatest variety of peptides.

As noted hereinabove, murine heavy chains complexed with human β-2 microglobulin were stable at temperatures approximately 6-8 degrees higher than if complexed with murine $\beta 2$. It was also observed that the stabilities imparted by peptide and xenogeneic β-2 microglobulin are additive. A large increase in the thermostability of the Class I molecules occurs if 8-9

mers are used, as compared to 12-25 mers; indeed, the difference between the stabilization imparted by the 8-9 mers compared with the larger peptides might be even greater than what was observed previously, for even though the peptides have been purified by HPLC, it is likely that there is some contamination of the larger peptides by 8-9 mers.

The thermostability of a Class I molecule is apparently dependent on: (1) the origin of β-2 microglobulin; (2) the presence of peptide; and (3) the length and sequence of this peptide.

Previous work (U.S. Patent No. 5,314,813 to Peterson et al.; Jackson et al., PNAS USA 89: 12117-12121 (1992)) has shown that Class I MHC heavy chains can bind peptide either alone or when they are associated with β-2 microglobulin. Surface expression of peptide-loaded human Class I MHC, however, appears to be best facilitated by loading the molecules with peptide after the heavy chains have complexed with β-2 microglobulin.

1. Expression of Human MHC

Once we determined that the thermostability of a Class I molecules is dependent on the origin of β-2 microglobulin, the presence of peptide, and the length and sequence of this peptide, we utilized this information in the creation of cell lines capable of specifically activating $CD8^+$ cells via the expression of peptide-loaded human Class I MHC molecules.

Thermolability appears to be an inherent property of Class I molecules; it has presumably evolved to ensure that Class I molecules which contain either no peptide or a peptide of poor binding properties (that confers little thermostability) self-destruct. In this way, the cell minimizes the number of empty Class I molecules on its surface, for such a situation would presumably be dangerous in that exogenously derived

peptides could be bound and presented. Human Class I molecules expressed in insect cells with human β2 are not stable to extended incubation at 37 °C; neither are human Class I molecules expressed in the mutant cell line T2 which has been shown to be deficient in peptide loading onto the Class I molecules (Hosken and Bevan, Science 248: 367-70 (1990); Cerundolo, et al., Nature 345: 449-452 (1990)). Thus, it seems that the affinity between the heavy chain and β-2 microglobulin has been carefully conserved through co-evolution of the molecules such that empty Class I molecules, or those carrying poorly-binding peptides, self-destruct at the body temperature of the "host" organism.

Human Class I MHC molecules were expressed in S2 cells. Cell lines co-expressing human β-2 microglobulin and HLA A2.2Y, HLA A2.1, HLA B7, or HLA B27 were established using previously-described methods. Briefly, cDNAs encoding the above proteins were cloned into the *Drosophila* expression vector pRmHa-3 and cotransfected with a human β-2 microglobulin-containing plasmid and phshsneo plasmid into S2 cells via methods disclosed herein. Three to four weeks later, the population of G418-resistanT-cells was diluted 1:5 with fresh selection media. Once a healthy growing population of cells was obtained, $CuSO_4$ was added to an aliquot of cells and 24 hours later, cells were analyzed via flow cytometry using a monoclonal antibody W6/32 (ATCC HB95, Bethesda, MD) which recognizes a monomorphic determinant of human Class I heavy chains when they are in association with β-2 microglobulin. (See Barnstable, et al., Cell 14: 9 (1978).) High levels of surface expression of each of the human Class I molecules were induced by the addition of $CuSO_4$ (data not shown). These stable populations were sorted for high expressing cells using cytofluorimetry as described below. It is these

sorted populations of cells which were used for all subsequent experiments.

Twenty-four hours prior to FACS analysis, $CuSO_4$ is added to the stably transfected S2 cells (3-4 X 10^6 cells/ml) to a final concentration of 1mM, thereby "switching on" expression from the transfected genes. Cells are plated out in 24-well cluster dishes (2 ml per well). Eight hours prior to FACS analysis, the $CuSO_4$ medium is replaced with fresh medium (1 ml) with or without peptide at a concentration of 50μg/ml. 37 °C temperature challenges are carried out by transferring the dishes onto a flat surface in a 37 °C room at various time intervals prior to harvesting the cells for analysis.

To analyze surface expression of Class I MHC on the S2 cells, aliquots of cells (5 X 10^5) are transferred into tubes on ice, collected by centrifugation (1,000 X g for 4 minutes), resuspended in 3 ml of PBS/1% BSA, 0.02% sodium azide, collected by centrifugation and resuspended in PBS/BSA (0.5 ml) containing the appropriate primary antibody (ascites fluids Y3, 28:14:8S, 30.5.7, W6/32, diluted 1:200). Rabbit antisera are diluted 1:500 and B22.293 hybridoma supernatant is used directly. After a one hour incubation on ice, cells are washed twice in 3 ml of PBS/BSA and resuspended in 0.5 ml of PBS/BSA containing FITC labelled secondary antibody (Cappell, Durham, NC) and 1 ng/ml propidium iodide. After a 30 minute incubation on ice, cells are washed once with PBS/BSA and resuspended in this buffer at a concentration of 1 X 10^6/ml. Samples are then analyzed by FACS 440 (Becton Dickinson). Dead cells stained with propidium iodide, are excluded by including a live gate in the analysis.

For cell sorting, the same procedure outlined above is used, except that all staining operations are

carried out in a sterile hood. Solutions, including antibodies, are filter-sterilized, and Schneider media or Excell 400 is used in place of PBS/BSA. Cells that specifically bound the primary antibody are sorted using a Becton Dickinson cell sorter. Sorted cells (2-8 X 10^5) are washed once in medium before plating out at a concentration of 2 X 10^5 cells/ml.

F. Loading of Membrane-Bound Empty MHC Molecules by *in vitro* Incubation with Peptides

In order to demonstrate that the human Class I molecules expressed on the surface of the *Drosophila* cells were empty, the cells were incubated at 37 °C for two hours and the cell surface expression was analyzed by cytofluorimetry. The surface expression of both HLA B27 and A2.1 is greatly reduced if cells are incubated at 37 °C for 2 hours; however, preincubating the cells in HIV peptides known to bind to the Class I molecules affords significant thermal stability to the Class I, while peptides that do not bind have little effect (see Figure 4). (A 9-amino acid peptide ILKEPVHGV (SEQ ID NO 42) from the POL protein of HIV binds and stabilizes HLA A2.1. A nine-amino-acid peptide from the Vpr protein of HIV binds and stabilizes B27 (FRIGCRHSR; SEQ ID NO 41). These data show that the human Class I molecules expressed on the surface of *Drosophila* cells are empty and can be stabilized by binding specific HIV peptides.

Figures 4 and 5 show peptide-induced thermostabilization of HLA B27 and HLA A2.1 expressed on the surface of *Drosophila* cells by HIV peptides. *Drosophila* cells expressing either HLA B27 or A2.1 were incubated with peptides where indicated and then either maintained at 28 °C or incubated at 37 °C for two hours prior to analysis of the surface expression of the Class I molecules by use of the antibody W6/32 (from ATCC HB95) and cytofluorimetry. The mean fluorescence of each

cell population is shown plotted against the incubation conditions The HIV POL peptide (ILKEPVHGV, SEQ ID NO 42) stabilizes A2.1 but not B27 (Figure 4), while the HIV Vpr peptide (FRIGCRHSR, SEQ ID NO 41) stabilizes B27, but not A2.1 (Figure 5).

Example 2

Preparation of Synthetic Antigen-Presenting Cells

A. Osmotic Loading

Osmotic loading of SC2 and 3T3 cells with ovalbumin protein was carried out as described by Moore, et al., Cell 54: 777-785 (1988). The assay procedure is as follows. In a 96-well dish, 1 X 10^5 *Drosophila* cells (with or without peptide/protein loaded) or 3T3 cells were cocultured with 1 X 10^5 B3/CD8 T-cell hybridoma cells in 200μl of RPMI media supplemented with 10% fetal bovine serum. After 24 hours of incubation, 100μl of the supernatant from these cultures was added to 100μl of RPMI containing 5,000 CTLL cells. The cells were cocultured for 24 hours at 37 °C when 1μCi of ^{3}H thymidine (Amersham) was added. After a further incubation of 15 hours at 37 °C, the incorporation of radiolabel into the CTLL cells was determined by scintillation counting.

Assays conducted with murine MHC also verified that the insect cells are capable of loading peptide onto the Class I molecules. Cells expressing as few as 200-500 MHC molecules containing a particular antigen can be detected by a T-cell. As the *Drosophila* cells do not accumulate chromium, an antigen presentation assay based on B3/CD8, a T-cell hybridoma, was used. B3/CD8 is a hybridoma between B3, cytotoxic T-cell specific for ovalbumin peptide 253-276 presented by H-2K^b Class I molecules, and CD8- bearing IL-2-secreting cell line (see Carbone, et al., supra, 1989). Upon antigenic stimulation, B3/CD8 produces IL-2, measured by ^{3}H

thymidine incorporation in IL-2-dependent T-cell line CTLL (Gillis, et al., J. Immunol. 120: 2027 91978)). Thus, by measuring the amount of IL-2 produced, one can assay for T-cell recognition.

In order to provide an intracellular pool of ovalbumin protein from which OVA peptides can be derived, ovalbumin (Sigma Chem. Co., MO) was osmotically loaded into the cells as described by Moore, et al, supra (1988). Immediately after loading, the cells were mixed with the T-cell hybridoma. After two days' incubation, the medium was removed and assayed for IL-2. The amount of IL-2 was determined by the ability of the medium to support the growth of the IL-2-dependenT-cell line CTLL (Gillis, et al., supra, 1978), and growth was quantitated by the amount of radioactive thymidine incorporated into the cells.

S2 or 3T3 cells transfected with $K^b/\beta 2$ were incubated with ovalbumin protein (OvPro) or ovalbumin peptide, OVA 24 (OvPep) in isotonic (Iso) or hypertonic (Hyp) media. (Murine cell line BALB/3T3 is available from the ATCC under accession number CCL 163.) After treatment, cells were cocultured with the T-cell hybridoma B3/CD8. B3/CD8 is a T-cell hybridoma between B3 (Carbone, et al., J. Exp. Med. 169: 603-12 (1989)), cytotoxic T-cell specific for ovalbumin peptide 253-276 presented by H-$2K^b$ Class I molecules, and CD8- bearing IL-2-secreting cell line. Upon antigenic stimulation, B3/CD8 produces IL-2, measured by ^3H thymidine incorporation in IL-2-dependent cell line CTLL (Gillis, et al., J. Immunol. 120: 2027 91978)). Thus, by measuring the amount of IL-2 produced, one can assay for T-cell recognition. The supernatant from the cocultures were analyzed for IL-2 by ^3H thymidine incorporation by the IL-2-dependent cell line CTLL (ATCC No. TIB 214).

The amount of ^3H thymidine incorporated is plotted against the initial cell treatments.

It can be seen in Figure 6 that the T-cells responded well to the *Drosophila* cells if the ovalbumin peptide was added to the culture medium, but no recognition occurred if the cells were loaded with the ovalbumin protein. The MHC Class I molecules expressed on the cell surface of the insect cell are fully functional in that they can bind peptide if it is added to the culture medium and can present it in the correct context for it to be recognized by a T-cell.

B. Optimization of *In Vitro* Conditions

For the optimization of *in vitro* conditions for the generation of specific cytotoxic T-cells, the culture of *Drosophila* cell stimulator cells is preferably maintained in serum-free medium (e.g. Excell 400). *Drosophila* cell stimulator cells are preferably incubated with >20μg/ml peptide. The effector:stimulator ratio (lymphocyte:*Drosophila* cell ratio) is preferably in the range of about 30:1 to 300:1. The maximum specific $CD8^+$ is generally observed after five days of culture. The culture of target cells for killing assay is preferably maintained in a serum-free medium.

Example 3

Stimulation of Proliferation and Differentiation of Armed Effector T-Cells

We have found that *Drosophila* S2 cells transfected with MHC class I molecules and specific assisting molecules are able to stimulate primary responses from T-cells in vitro. We present data below in this example from a mouse model system. In this example, constructs coding for mouse MHC class I (L^d), β2 microglobulin, specific assisting molecules were used and tested with $CD8^+$ cells from lymph nodes of T-cell receptor transgenic mice.

The data in Figure 7 provides evidence that the transfected *Drosophila* S2 cells express the protein products of the transfected murine genes. Flow cytometry using a fluorescence-activated cell sorter (FACS) and fluorescently labelled antibodies were used to demonstrate the expression of L^d (MHC molecule which includes heavy chain and $\beta 2$) and the specific assisting molecules B7.1 (CD80) and ICAM-1 (CD54) molecules by transfected *Drosophila* S2 cells. Transfected cells were separated with a FACS to obtain cells expressing L^d molecules and were then maintained in vitro.

The transfection of *Drosophila* S2 cells is summarized in Table 2. The data show L^d, B7.1 and ICAM-1 expression measured by flow cytometry on the cell lines after induction with $CuSO_4$. It is apparent that, relative to the control antibody (ctr Ab), all of the transfectants express L^d molecules on the cell surface. Likewise, cells cotransfected with L^d and B7.1 (L^d.B7) express B7.1 but not ICAM-1, whereas cells cotransfected with L^d and ICAM-1 (L^d.ICAM) express ICAM-1 but not B7.1; triple transfection with L^d, B7.1 and ICAM-1 (L^d.B7.ICAM) led to expression of all three molecules.

Using a standard tissue culture system (Cai, Z. and Sprent, J. (1994) J. Exp. Med. 179: 2005-2015), doses of 5 x 10^4 purified $CD8^+$ 2C lymph node (LN) cells were cultured at 37 °C with doses of 3 x 10^5 transfected fly cells ± peptides (10 μM final concentration). Peptides were synthesized by R. W. Johnson Pharmaceutical Research Institute (Sykulev, et al. (1994) Immunity 1: 15-22. Proliferative responses were measured by adding ^{3}HTdR (1 μCi/well) 8 hours prior to harvest. IL-2 production was measured by removing supernatants from the cultures at 48 hours and adding 50 μl supernatant to an IL-2 responsive indicator cell line (CTLL); proliferation of the indicator line was measured by addition of ^{3}HTdR.

The data shown in Table 2 are the means of triplicate cultures. The transfected *Drosophila* S2 cells die rapidly at 37 °C and fail to incorporate ^{3}HTdR at this temperature.

The data in Table 2 demonstrate that the transfectants are able to stimulate primary responses of mouse T-cells.

Table 2. Capacity of transfected fly cells to stimulate primary proliferative responses and IL-2 production by CD8$^+$ lymph node cells from 2C T-cell receptor transgenic mice.

TABLE 2
^{3}HTdR incorporation (cpm x 10^3) with transfected fly cells expressing:

Assay	Peptides added	L^d	L^d + B7.1	L^d + ICAM-1	L^d + B7.1 + ICAM-1	L^d + B7.1 combined with L^d + ICAM-1
Proliferation (Day 3)	-	0.2	0.1	0.3	0.2	.
	p2Ca	0.2	0.3	1.5	142.0	1.5
	QL9	0.2	60.9	73.9	263.7	132.9
IL-2 Production (Day 2)	-	0.3	0.2	0.1	1.2	.
	p2Ca	0.2	0.2	0.1	64.6	0.3
	QL9	0.1	0.4	0.2	158.6	0.5

The 2C T-cell receptor (TCR) is strongly reactive to L^d molecules complexed with certain peptides, e.g. p2Ca (SEQ ID NO 46) or QL9 (SEQ ID NO 47). These two peptides have moderate to high affinity for soluble L^d molecules, 4 x 10^{-7} M^{-1} for p2Ca, and 4 x 10^{-6} M^{-1} for QL9 (Sykulev. et al.). When complexed to soluble L^d molecules, the two peptides also have high binding affinity for soluble 2C TCR molecules. However, in both TCR binding and L^d binding, the QL9 peptide clearly has a higher affinity than the p2Ca peptide.

Table 2 shows that proliferative responses and IL-2 production by the responder 2C cells to the weaker peptide, p2Ca, requires that the stimulator L^d-transfected cells coexpress both B7.1 and ICAM-1; a mixture of cells expressing either L^d + B7.1 or L^d + ICAM-1 is nonstimulatory. By contrast, with the stronger peptide, QL9, L^d.fly cells expressing either B7 or ICAM elicit clearly-significant responses, although combined expression of B7 and ICAM generates much higher responses. In contrast to these findings on T-cell proliferation, IL-2 production in response to the QL9 peptide requires joint expression of B7 and ICAM; expression of these molecules on separate cells is ineffective.

The results show that Drosophila cells transfected with murine class I molecules and costimulatory molecules induce murine T-cells to mount primary proliferative responses and lymphokine (IL-2) production in response to peptide antigens. The system is also applicable to human T-cells and could be used to stimulate unprimed (or primed) T-cells specific for tumor-specific antigens in vitro; in vivo infusion of clonally-expanded T-cells specific for tumor-specific antigens might be therapeutic for patients with cancer. Infusion of T-cells specific for viral antigens would be useful in patients with viral infections, e.g. HIV.

Example 4

Immobilization of Biotinylated MHC Molecules on Avidin-Coated Red Blood Cells

NHS-LC-biotin, neutravidin and biotin-BMCC were purchased from Pierce (Rockford, IL). Sheep red blood cells were obtained from the Colorado Serum Company (Denver, CO). *Drosophila* S2 cells expressing L^d and recombinant L^d were prepared as described in Examples 1 and 2. Monoclonal antibodies 30.5.7 (anti-L^d) and 1B2

(anti-clonotypic antibody to the 2C T-cell receptor) were used as hybridoma cell culture supernatants.

The protocol used is described by Muzykantov and Taylor (Anal. Biochem. (1994) 223, 142-148). Briefly, SRBC were washed 4 times in phosphate buffered saline (PBS), biotinylated using NHS-LC-biotin, washed again 4 times in PBS, incubated with neutavidin, and finally washed 4 times and stored at 4 °C in PBS containing 3% fetal calf serum and 0.02% sodium azide.

Recombinant L^d was biotinylated using biotin-BMCC, a maleimide-coupled biotin which reacts with thiol groups. L^d displays a free thiol group, the side chain of cystein 121, which is not in the peptide binding site. Biotinylation was performed as recommended by the manufacturer. Unreacted biotin was removed using Centricon 10.

Biotinylated L^d was immobilized by incubation at a final concentration of 0.2 mg/ml with avidin-coated SRBC for 30 minutes followed by washing in DMEM containing 10% fetal calf serum. SRBC with attached L^d were used immediately.

T-cells expressing the 2C TCR transgene from lymph nodes of mice were purified by magnetic depletion. Purified T-cells were consistently 97-98% positive for staining in flow cytofluorometry using the anti-clonotypic antibody 1B2.

Immobilization of biotinylated L^d on avidin-coated SRBC was done as indicated above. Attachment was assessed using flow cytofluorometry using anti-L^d antibody 30.5.7.

A typical experiment is represented in Figure 8. The negative control (cells minus antibody) is shown in dotted lines. The filled peak comprises cells labeled with fluorescent antibody. 99.78% of the cells were labeled. Fluorescence intensity was in the same

range than the highest levels of intensity that we observed for L^d on synthetic antigen-presenting cells.

K^b (MHC molecule which includes heavy chain and $\beta 2$) was also biotinylated using the same procedure. We could immobilize biotinylated K^b on avidin-coated SRBC as assessed by flow cytofluorometry (Figure 9). 99.88% of the cells were labeled.

Rosetting experiments verified that the attached MHC molecules interacted functionally with T-cells. *Drosophila* S2 cells expressing L^d, L^d-coated SRBC were incubated with QL9 peptide (0.02 mM) or an irrelevant peptide (MCMV, 0.02mM) for 30 minutes on ice; 2C+ T-cells were then added, the proportion being 10 2C+ T-cells for 1 *Drosophila* S2 cell, or 10 SRBC for 1 2C+ T-cell; the mixture was pelleted and kept on ice for at least 30 min. Cells were then carefully resuspended and rosettes were counted, a rosette being a *Drosophila* S2 cell bound to at least 3 2C+ T-cells, or a 2C+ T-cell bound to at least 3 SRBC. Rosettes were observed in all cases. Typically, 30-40% of the lymphocytes were included in rosettes when QL9 peptide was added. No rosette was observed in the presence of the irrelevant peptide, although occasional attachment of a few single cells was observed.

These examples describe a new method to immobilize high amounts of MHC class I molecules on various surfaces (fly cells, red blood cells, latex beads) in native conformation as judged by monoclonal antibody binding and rosetting experiments (T-cell receptor binding). This method can be extended to other synthetic surfaces including artificial phospholipid membranes. Phosphatidylethanolamine as well as avidin-coupled phospholipids are particularly relevant to our studies. These phospholipids are commercially

available from Lipex Biomembrane Inc., Vancouver, BC, Canada.

Example 5

Immobilization of Biotinylated MHC Molecules on Avidin-Coated Latex Beads

Six micron diameter latex sulfate beads were purchased from Interfacial Dynamics Corporation (Portland, OR) and biotinylated according to the protocol described in Example 4.

Avidin-coated latex beads were prepared using a 1% suspension of the latex beads incubated in PBS containing 1mg/ml of neutravidin for one hour at room temperature. An equal volume of PBS containing 10% fetal calf serum was then added. After one hour of incubation at room temperature, the beads were washed 3 times and used for binding of recombinant biotinylated L^d.

Recombinant biotinylated L^d was immobilized by incubation at a final concentration of 0.2 mg/ml with avidin-coated latex beads for 30 minutes followed by washing in DMEM containing 10% fetal calf serum. SRBC with attached L^d were used immediately.

Rosetting experiments verified that the attached MHC molecules on latex beads interacted functionally with T-cells. *Drosophila* S2 cells expressing recombinant L^d and L^d-coated latex beads were incubated with QL9 peptide (0.02 mM) or an irrelevant peptide (MCMV, 0.02mM) for 30 minutes on ice; 2C+ T-cells were then added, the proportion being 10 2C+ T-cells for 1 *Drosophila* S2 cell, or L^d-coated latex beads for 1 2C+ T-cell; the mixture was pelleted and kept on ice for at least 30 min. Cells were then carefully resuspended and rosettes were counted, a rosette being a *Drosophila* S2 cell bound to at least 3 2C+ T-cells, or a 2C+ T-cell bound to at least 3 latex beads. Rosettes were observed in all cases. Typically, 30-40% of the lymphocytes were

included in rosettes when QL9 peptide was added. No rosette was observed in the presence of the irrelevant peptide, although occasional attachment of a few single cells was observed.

Example 6

Immobilization and Detection of Recombinant Protein Bound to Various Solid Supports Such as Plastic Microwell Plates

The MHC molecules were immobilized by direct binding to microtiter plates (Corning) and detected as follows:

MHC K^b molecules were diluted to desired concentration in PBS, e.g. 0.001mg/ml for 100 ng /well. 100μl of diluted K^b was added to each well on the plastic microtiter plate. The plate was incubated for 1 hour at room temperature. After incubation, the plate was washed once with PBS and 200μl 2% bovine serum albumin (BSA) in PBS + (.05%) and Tween (PBST) was added, and incubated for another hour at room temperature. The plate was washed three times with PBST and biotinylated anti-K^b mAb was added (1:2500) in 2% BSA in PBS. The plate was incubated another hour at room temperature and washed three times with PBST. Avidin conjugated HRP was added (1:2500) in 2% BSA in PBS. Following another hour of incubation at room temperature, the plate was washed three times with PBST and H_2O_2 or thophenyldiamine was added. The reaction was stopped with H_2SO_4. Reaction product was detected colorimetrically at 490 nm.

Figure 10 shows the results of detecting the presence of MHC K^b molecules using three different monoclonal antibodies.

Recombinant MHC K^b molecules can alternatively be bound through biotin-avidin linked interactions with the substrate. In this embodiment, the microwell plates were coated with 100μl avidin diluted in PBS to a

concentration of 0.001mg/ml. Excess avidin was removed by a PBS wash. The above procedure for presenting and detecting K^b binding followed.

Recombinant MHC molecules may alternatively be immobilized by a linkage based on a poly-histidine tag added to the MHC interacting with the nickel bound to the substrate.

The above procedure for binding and detection is followed using nickel chelate coated microwell plates (Xenopore) and recombinant MHC molecules with a poly-histidine tag expressed using vector pRmHa/His_6 described above.

Example 7

Direct Binding of Peptide to Soluble, Empty Class I MHC Molecules *In Vitro*

A. Procedures

H-$2K^b$: prepared as described above in Example 1.B.

H-$2K^b$ Sol: K^b sol cDNA is a derivative of K^b, encoding the extracellular portion of the Class I MHC molecule. K^b sol cDNA may be produced by PCR according to known methods, such as those described in Ennis, et al., PNAS USA 87: 2833-7 (1990) and Zemmour, et al., Immunogenetics 33: 310-20 (1991). Specifically, cDNA encoding a truncated K^b molecule with a stop codon inserted at the end of the alpha 3 domain at amino acid position +275 is excised from the pCMU expression plasmid as a Bam HI fragment and cloned into pRmHa-3 as K^b cDNA. The K^b sol cDNA is a derivative of the complete K^b cDNA (see above) which is used as a template in a PCR reaction using a 5′ oligonucleotide that encompassed the Sty I site, and the following 3′ oligonucleotide:

5′ ATATGGATCCTCACCATCTCAGGGTGAGGGGC 3′

(SEQ ID NO 43)

The resulting PCR fragment is blunt-end cloned into the Sma I site of pBS (Stratagene, La Jolla, CA), sequenced, and the remaining 5′ sequence of K^b cloned into the Sty I site. A cDNA encoding the complete K^bsol protein could be obtained as a Bam HI restriction fragment.

H-2D^b and H-2L^d are prepared as discussed in Example 1.B. above.

The cDNAs encoding K^b $\alpha 1\alpha 2\alpha 3$ domains (274 residues) and murine β-2 microglobulin (99 residues) were respectively cloned into the unique Bam HI site of an expression vector harboring the metallothionein promoter pRMHa-3 (Bunch, et al., Nucleic Acid Res. 16: 1043-1061 (1988)). *Drosophila* S2/M3 cells were transformed with these recombinant plasmids in addition to plasmid phshsneo (containing a neomycin-resistance gene) by the calcium-phosphate precipitation method described previously. The transformed cells selected against neomycin-analog antibiotics G418 were grown at 27 °C in serum-free medium and soluble heavy-chain K^b and β-2 microglobulin were co-expressed by the addition of 0.7mM $CuSO_4$.

The soluble, assembled heterodimer of K^b was purified from the culture supernatants by affinity chromatography using anti-K^b monoclonal antibody Y3, followed by ion-exchange chromatography on a Pharmacia Mono Q FPLC column according to the instructions of the manufacturer (Pharmacia, Piscataway, NJ). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the K^b preparation followed by staining with Coomassie blue showed only one band of relative molecular mass (Mr) at about 32,000 and one band of Mr at about 12,000 with no detectable impurities. The highly-purified K^b was dialyzed against phosphate-buffered saline (PBS), filter-sterilized, and used for further study.

Extinction coefficient of the soluble K^b ("K^bsol") protein (43.2 kDa) is 69,200 $M^{-1}cm^{-1}$ at 280 nm.

The purified K^b sol (0.3μM) in PBS with or without 1% TX-100 were exposed to varying temperatures (i.e., 4°, 23°, 32°, 37°, 42°, and 47°C) for one hour. The proteins were then immunoprecipitated by incubating with the monoclonal antibody Y3 and protein A sepharose beads (Pharmacia, Piscataway, NJ) at 4 °C for two hours, respectively. The samples were analyzed by 12.5% SDS-PAGE, followed by staining with Coomassie blue. The two thick bands on the gel are heavy and light chains of antibody Y3. In another procedure, K^bsol (0.3μM) were incubated with 50μM of peptides in PBS at 23 °C for two hours to allow for K^bsol-peptide complex formation. After the addition of 1% TX-100, the samples were exposed to 12 °C, 37 °C, or 47 °C temperatures for one hour. The complexes were immunoprecipitated and analyzed by SDS-PAGE as described above. In a third procedure, K^bsol (2.7μM) were incubated with 50μM of OVA-8, VSV-8 or SEV-9 peptides, respectively, at 23 °C for two hours. The samples were applied on a 5% polyacrylamide IEF gel. IEF was run from pH 5-7 and the gel was stained with silver.

Next, VSV-8 peptide was radioiodinated using the chloramine-T method (Hunter, et al., Nature 194: 495-6 (1962)) and free ^{125}I was removed by C_{18} column (OPC cartridge, Applied Biosystems, Foster City, CA). The labelled peptide was further purified by C_{18} reverse-phase HPLC. After elution, the labelled peptide was lyophilized and resuspended in PBS.

The specific activity of [^{125}I]VSV-8 (about 250 Ci/mmole) was determined spectrophotometrically by using extinction coefficient of tyrosine at 274nm (1420 $M^{-1}cm^{-1}$). First, K^bsol (0.5μM) was mixed with [^{125}I]VSV-8 (1.5nM) and unlabelled VSV-8 (50nM) at 23 °C for 16 hours to allow for complex formation. A portion of the sample

was analyzed by gel filtration (Superose 12, Pharmacia, Piscataway, NJ) in PBS. After elution, radioactivity contained in each fraction (0.05ml) was measured. Protein was monitored by absorbance at 280nm.

In a second procedure, [^{125}I]VSV-8 (0.39nM) was mixed with various concentrations of K^bsol in PBS containing 1% bovine serum albumin (BSA). After incubation at 23 °C for 2-16 hours, K^bsol-peptide complexes were separated from free peptide by small gel filtration (Bio-Gel P30, BioRad, Richmond, CA) in PBS. P30 gel filtration permitted over 95% separation of bound and free peptide within about 5 minutes. Radioactivity of bound and free peptides was measured and the data were analyzed by linear regression. At maximal levels of K^bsol offered, about 65% of the total labelled peptides were bound. This maximal binding capacity of labelled peptide to K^bsol protein deteriorated over time, presumably due to radiation by ^{125}I bound to VSV-8.

In a third procedure, each sample contained 0.39nM of [^{125}I]VSV-8 (about 18,000 cpm), unlabelled peptides at the indicated concentration, and 30nM of K^bsol that gives about 50% of the [^{125}I]VSV-8 binding in the absence of unlabelled peptide at a final volume of 72μl. All components were dissolved and diluted in PBS containing 1% BSA. After incubation for 2-16 hours at 23 °C, 50μl samples were analyzed by P30 gel filtration as described above. The dissociation constants for unlabelled peptides were determined from molar concentrations of [^{125}I]VSV-8 and unlabelled peptides giving 50% inhibition of [^{125}I]VSV-8 binding to K^bsol as described. (See Muller, et al., Meth. Enzymol. 92: 589-601 (1983).)

K^bsol (0.3μM) and [^{125}I]VSV-8 (0.39nM) were then incubated at 4° C, 23 °C, and 37 °C, and the association was determined at various times by P30 gel filtration.

Murine β-2 microglobulin was added, when necessary, before the incubation at the indicated concentration. The murine β-2 microglobulin was prepared by affinity chromatography using anti-β-2 microglobulin polyclonal antibody K355 from culture supernatants of the recombinant *Drosophila* cells. (See also Logdberg, et al., Molec. Immun. 14: 577-587 (1979).) In another experiment, K^bsol (0.3μM or 1.8μM) and [^{125}I]VSV-8 (2.4nM) were incubated at 23 °C for two hours, and the peptide-K^bsol complexes were isolated by P30 gel filtration. The samples contained very small amounts of [^{125}I]VSV-8 and K^bsol complexes (at the maximum, 2.4nM) and empty K^bsol at final concentration of about 50 to 300nM. To some samples, 3μM of β-2 microglobulin, 3μM of β-2 microglobulin plus 20μM of unlabelled VSV-8, 20μM of unlabelled VSV-8, or 1% TX-100 were added. The samples were incubated for various times at 37 °C and the degree of dissociation was determined by passage over P30 columns.

B. Discussion

Class I MHC molecules present antigenic peptides to cytotoxic T lymphocytes. Direct binding of peptide to Class I molecules *in vitro* has been hampered by either the presence of previously bound peptides at the binding site (Chen and Perham, Nature 337: 743-5 (1989)) or the lack of binding specificity. (See, e.g., Frelinger, et al., J. Exp. Med. 172: 827-34 (1990); Choppin, et al., J. Exp. Med. 172: 889-99 (1990); Chen, et al., J. Exp. Med. 172: 931-6 (1990).) *In vitro* analysis of peptide binding to soluble, empty Class I molecules purified from *Drosophila* cells transformed with truncated H-2K^bsol and murine β-2 microglobulin genes is disclosed herein. The results demonstrate that peptide binding is very rapid and naturally processed peptides (octapeptides; see, e.g., Van Bleek, et al., Nature 348:

213-6 (1990); Falk, et al., Nature 351: 290-6 (1991)) have the highest affinities to K^bsol of the nanomolar range and indicate that K^bsol complexed with octapeptides are stable, whereas those complexed with slightly shorter or longer peptides are short-lived. Interactions between free heavy chain and β-2 microglobulin is basically reversible in the absence of detergent. Peptides spontaneously bind to empty Class I molecules without dissolution of β-2 microglobulin. However, excess β-2 microglobulin apparently promotes the binding of peptide to empty Class I as a consequence of reassociation of free heavy chain with β-2 microglobulin under conditions where the heterodimers are unstable.

Soluble H-2K^b molecules (composed of the α1α2α3 domain of heavy chain) and murine β-2 microglobulin, were purified from the culture supernatants of *Drosophila* cells which were concomitantly transformed with the truncated heavy chain and β-2 microglobulin genes. Preliminary examinations suggested that *Drosophila* cells express Class I MHC molecules devoid of endogenous peptides on the cell surface. Some of the properties of empty Class I molecules include the observation that they are less stable at 37 °C and their structure is stabilized by the binding of peptide. (See, e.g., Schumacher, et al., Cell 62: 563-7 (1990); Ljunggren, et al., Nature 346: 476-80 (1990).) To confirm that purified soluble K^b are also empty, their thermal stability in detergent-free solution was examined. Surprisingly, the proteins heated for one hour at 47 °C were well recovered by immunoprecipitation using a conformational antibody, Y3. This unexpected result led us to add detergent, 1% Triton X-100 (polyoxyethylene (9) octyl phenyl ether), to the protein solution, since similar experiments to test the stability of Class I molecules have always been conducted in detergent lysates

(See Schumacher, et al., cited *supra*). The results obtained in the presence of detergent show that the purified K^bsol is now unstable at 37 °C. This and other lines of evidence suggest that K^bsol heterodimer disassembles into the heavy chain and β-2 microglobulin at elevated temperatures and that detergent may prevent β-2 microglobulin from reassociating with dissociated free heavy chain (see below). Second, the possibility of stabilizing purified K^bsol with peptides was studied. The results of the first-described examination demonstrated that the proteins can be stabilized only when they are mixed with octapeptide (vesicular stomatitis virus nucleocapsid protein [VSV-8], see Table 3 below) which is shown to be a naturally processed peptide (see Van Bleek, et al., cited *supra*). These observations are consistent with the characteristics of empty Class I molecules mentioned above.

Independent support that the purified K^bsol molecules are empty is provided by isoelectric focusing (IEF) under native conditions (data not shown). The soluble K^b purified from *Drosophila* cells exhibited a much simpler pattern than HLA-A2 molecules purified from human lymphoblastoid cell lines (see Figure 3 in Silver, et al., Nature 350: 619-22 (1991)). The complicated pattern of HLA-A2 on IEF is presumed to be the result of the presence of heterogeneous peptides bound to the molecules. The simple band of purified K^bsol indicates the absence of endogenous peptides. In addition, the incubation of K^bsol with antigenic peptides caused the distinct shifts of band on IEF gel, reflecting the change in isoelectric point of K^bsol due to the peptide binding. It should be noted that such band-shifting was not observed in HLA-A2 molecules when they were simply mixed with peptides, unless HLA-A2 are incubated with peptides in "reconstituting conditions" after removal of

previously bound endogenous peptides. Taken together, these observations on native IEF also indicate that soluble K^b purified from *Drosophila* cells are empty.

The association of ^{125}I-labelled VSV-8 with K^bsol was demonstrated by gel filtration (not shown). The radioactivity of high molecular weight materials corresponds to peptide-K^bsol complexes, while that of low molecular weight materials represents free peptides. Unlabelled VSV and ovalbumin (OVA) peptides could compete with the labelled VSV-8 (see below), arguing that [^{125}I]VSV-8 is bound specifically to K^bsol molecules. Reversed-phase HPLC revealed that K^b-bound [^{125}I]VSV-8 has the identical retention time to the input peptide. The binding to K^bsol of the labelled VSV-8 was saturable, exhibiting a dissociation constant (K_D) of about 33nM (not shown). From the x-axis of the Scatchard plot, it was noted that about 65% of the labelled VSV-8 is able to bind to K^b.

To determine affinities of various peptides to K^b, competitive radioimmunoassays (RIA) using [^{125}I]VSV-8 were carried out (data not shown). The inhibitory peptides used for the RIA are listed in Table 3. K_D for each peptide is summarized in Table 3 as well.

TABLE 3

Various Antigenic Peptides* Used in Present Studies

Code	Sequence	K_D (M)
VSV-7	GYVYQGL	5.3×10^{-8}
VSV-8	RGYVYQGL	3.7×10^{-9}
VSV-9N	LRGYVYQGL	7.3×10^{-9}
VSV-10N	DLRGYVYQGL	3.9×10^{-7}
VSV-9C	RGYVYQGLK	6.9×10^{-9}
VSV-10C	RGYVYQGLKS	2.1×10^{-8}
OVA-8	SIINFEKL	4.1×10^{-9}
OVA-9N	ESIINFEKL	8.9×10^{-8}

OVA-10N	LESIINFEKL	2.8×10^{-7}
OVA-9C	SIINFEKLT	1.1×10^{-8}
OVA-10C	SIINFEKLTE	1.4×10^{-8}
OVA-24	EQLESIINFEKLTEWTSSNVMEER	7.1×10^{-5}
SEV-9	FAPGNYPAL	2.7×10^{-9}

VSV-8: Vesicular stomatitis virus nucleocapsid protein 52-59 (Van Bleek, et al., Nature 348: 213-216 (1990))
OVA-8: Ovalbumin 257-264 (Carbone, et al., J. Exp.Med. 169: 603-12 (1989));
SEV-9: Sendai virus nucleoprotein 324-332 (Schumacher, et al., Nature 350: 703-706 (1991))
* All peptides were purified by C_{18} reversed-phase HPLC to exclude contaminating shorter peptides with different binding properties. The 3-letter code designations and SEQ ID NO for each peptide are given below.

VSV-7	GlyTyrValTyrGlnGlyLeu (SEQ ID NO 40, residue nos. 4-10)
VSV-8	ArgGlyTyrValTyrGlnGlyLeu (SEQ ID NO 40, residue nos. 3-10)
VSV-9N	LeuArgGlyTyrValTyrGlnGlyLeu (SEQ ID NO 40, residue nos. 2-10)
VSV-10N	AspLeuArgGlyTyrValTyrGlnGlyLeu (SEQ ID NO 40)
VSV-9C	ArgGlyTyrValTyrGlnGlyLeuLys (SEQ ID NO 44, residue nos. 1-9)
VSV-10C	ArgGlyTyrValTyrGlnGlyLeuLysSer (SEQ ID NO 44)
OVA-8	SerIleIleAsnPheGluLysLeu (SEQ ID NO 39, residue nos. 5-12)
OVA-9N	GluSerIleIleAsnPheGluLysLeu (SEQ ID NO 39, residue nos. 4-12)
OVA-10N	LeuGluSerIleIleAsnPheGluLysLeu (SEQ ID NO 39, residue nos. 3-12)
OVA-9C	SerIleIleAsnPheGluLysLeuThr (SEQ ID NO 39, residue nos. 5-13)

OVA-10C SerIleIleAsnPheGluLysLeuThrGlu (SEQ ID NO 39, residue nos. 5-14)

OVA-24 GluGlnLeuGluSerIleIleAsnPheGluLysLeuThrGlu-TrpThrSerSerAsnValMetGluGluArg (SEQ ID NO 39)

SEV-9 PheAlaProGlyAsnTyrProAlaLeu (SEQ ID NO 45)

The peptides of naturally processed size (8mer for VSV and OVA, and 9mer for sendai virus nucleoprotein [SEV]) had the highest and remarkably similar affinities from the range of 2.7 to 4.1 nM. this exceedingly high affinity of the natural peptides is consistent with recent observations. (See, e.g., Schumacher, et al., Nature 350: 703-6 (1991); Christnick, et al., Nature 352: 67-70 (1991).) However, peptides that were shorter or longer by as little as one or two residues lowered the affinity by a factor of from 2 to 100. This reduction of the affinity is even more drastic for a much longer peptide; i.e., the affinity of 24mer peptide (OVA-24) is more than 10,000-fold lower than that of OVA-8. These results help to explain why earlier reports using longer peptides claim the affinity of micromolar range. (See, e.g., Frelinger, et al. and Choppin, et al., both cited *supra*.) It is of particular interest that the extension of peptides at the carboxyl terminus is much less destructive of the affinity than extension at the amino terminus. According to the three-dimensional structure of HLA-A2, the peptide-binding groove is formed by two long α helices on the antiparallel β strands, and the cleft is about 25 angstroms long, which is proposed to accommodate an extended peptide chain of about eight residues (see, e.g., Bjorkman, et al., Nature 329: 506-12 (1987)). At one end of the cleft, the α1 and α2 helices come close together tightly, while at the other end, the cleft is fairly open. It is now speculated that both VSV and OVA peptide bind to the cleft in the same orientation

*and the carboxyl terminus of the peptides might interact with the relatively open end of the cleft so that the extension of peptide at the carboxyl terminus does not cause severe steric hindrance.

Examinations were then performed to determine the rate of peptide binding to K^b at 4 °C and 23 °C, respectively (not shown). Binding was very rapid, especially at 23 °C, with a half-time of about 5 minutes even in extremely low concentrations of labelled peptides (about 0.4nM). This contrasts with previous observations, which show a half-time of association of about two hours. (See, e.g., Choppin, et al., cited *supra*.) Again, only 65% of the total labelled peptide was able to bind. The addition of excess β-2 microglobulin did not affect the peptide-binding kinetics at such low temperatures that K^b heterodimer is stable (remained to be assembled). This implies that exchange of β-2 microglobulin is not a prerequisite for peptide binding; i.e., peptides can spontaneously bind to empty Class I molecules without dissociation of β-2 microglobulin. In contrast, excess free β-2 microglobulin apparently promotes peptide binding at 37 °C (data not shown). As the concentration of added β-2 microglobulin increased, more peptides bound to K^b molecules. Since empty K^b are unstable at 37 °C, a fraction of heterodimers must be dissociated to the heavy chain and β-2 microglobulin and thereby, the heterodimer must be in equilibrium with free heavy chain and free β-2 microglobulin. Then, the addition of β-2 microglobulin should shift the equilibrium toward the formation of heterodimer that can bind peptides. This view is supported by recent observations that there are substantial numbers of Class I free heavy chains on the normal cell surface and exogenously added β-2 microglobulin facilitates peptide binding to empty

Class I molecules on cells as a consequence of the reassociation of β-2 microglobulin with free heavy chain. (See, e.g., Rock, et al., Cell 65: 611-620 (1991); Kozlowski, et al., Nature 349: 74-77 (1991); Vitiello, et al., Science 250: 1423-6 (1990).)

The dissociation kinetics of peptide at 37 °C were then observed. Immediately after isolating [^{125}I]VSV-8 and K^b complexes by gel filtration, the samples containing either 50 or 300 nM K^b were exposed to 37 °C temperatures. Some samples were supplemented with 3μM β-2 microglobulin and/or 20μM unlabelled VSV-8, or 1% TX-100. The dissociation of labelled peptides from K^b was measured at various times (not shown). In the presence of a large excess of unlabelled peptides, the dissociation rate of peptide followed first-order kinetics with a half-time dissociation of about 36 minutes (a dissociation rate constant of 3.2×10^{-4} s^{-1}). This unexpected, relatively rapid dissociation of labelled peptide does not agree with some current views of stable peptide-Class I complexes. In fact, the results ascertained (not shown) demonstrated that K^b and VSV-8 complexes are stable. This discrepancy must arise from the 10-fold lower affinity of radiolabelled VSV-8 (33 nM) compared with that of unlabelled VSV-8 (3.7 nM).

The first-order kinetics were also observed when the detergent was added instead of unlabelled peptide, indicating that the detergent makes the peptide dissociation process irreversible. In contrast, the peptide dissociation profile did not follow the first-order kinetics in the absence of unlabelled peptide or the detergent. This suggests that the peptide association/dissociation is reversible and the binding of peptide is dependent on the concentration of heterodimer (compare the kinetics between 50 nM and 300 nM of K^b). This became more evident when excess β-2 microglobulin

was added. These results support the previous argument that interaction between the heavy chain, β-2 microglobulin and peptide are basically reversible at 37 °C, if not entirely, in the absence of detergent. It is probable that a detergent such as TX-100 may prevent β-2 microglobulin from reassociating with free heavy chain at 37 °C. This could reasonably explain why K^b once heated to elevated temperatures in the absence of detergent can be efficiently immunoprecipitated by conformational antibody (not shown). Interestingly, the addition of β-2 microglobulin did not suppress the peptide dissociation in the presence of excess unlabelled peptides, indicating that labelled peptides are released from the complexes without dissociation of β-2 microglobulin. It should be remembered, however, that the affinity of [^{125}I]VSV-8 is about 10-fold lower than that of the natural peptides. Therefore, this is not necessarily the case for the natural peptides.

The study using *in vitro* peptide-binding assay systems suggests that peptide binding to Class I molecules is a simple mass action and a ligand-receptor interaction. The approach used herein allows characterization of the peptide binding specificity to Class I molecules and of the interaction of peptide-Class I complexes with the T-cell receptor.

Example 8

Therapeutic Applications

A. Class I Molecule Bank

A reservoir or "bank" of insect cell lines may be established and maintained, with each cell line expressing one of the 50 to 100 most common Class I MHC heavy chain, a β-microglobulin molecule, as well as at least one assisting molecule. cDNAs encoding these proteins may be cloned based on HLA variants obtained from cell lines containing same -- e.g., via the

polymerase chain reaction (see Ennis, et al., PNAS USA 87: 2833-7 (1990)) -- and inserted into the appropriate vector, such as an insect expression vector, to generate cell lines expressing each HLA variant.

Testing according to the following protocol, for example, can be used to determine which peptides derived from the virus of choice bind the best to the different Class I MHC molecules. The various cultures may appropriately be labeled or catalogued to indicate which Class I MHC molecules are best for use with particular peptides. Alternatively, transient cultures may be established as needed. As discussed herein, after approximately 48 hours' incubation of a culture of insect cells with a vector, that culture is apparently capable of expressing empty MHC molecules which may be loaded with the peptide(s) of choice for the purpose of activating $CD8^+$ cells.

B. Preparation of "Special" Cell Lines

After HLA typing, if insect cell lines expressing the preferred HLA are not available, cDNAs encoding the preferred HLA and assisting molecules may be cloned via use of the polymerase chain reaction. The primers disclosed in section B.1. above (SEQ ID NO 1 through SEQ ID NO 12) may be used to amplify the appropriate HLA-A, -B, -C, -E, -F, or -G cDNAs in separate reactions which may then be cloned and sequenced as described in the methods disclosed in Example 1, section 1 above. DNA is then purified from the PCR reaction using a Gene Clean kit (Bio 101, San Diego, CA) and ligated directly into the Sma I site of pRmHa-3. Individual clones are isolated, the sequences verified, and stable *Drosophila* cell lines expressing the HLA established. Alternatively, a bulk population of recombinant plasmids may be grown in large scale and DNA purified by cesium chloride gradients. The purified DNA

is then used to transfect S2 cells using calcium phosphate precipitation techniques. After 24 hours, the precipitate is washed off the cells and replaced with fresh Schneider media containing 1mM $CuSO_4$. Forty-eight hours later, the bulk population of transiently transfected cells is used for *in vitro* activation of $CD8^+$ after incubation with syngeneic peptides or protease digests of specific proteins.

Stable cell lines expressing the cloned HLA may then be established. Alternatively, a population of insect cells transiently expressing a bulk population of cloned recombinant molecules from the PCR reaction may be used for *in vitro* $CD8^+$ activation.

It is also possible to activate haplotype-specific CD8s *in vitro* using insect cells expressing Class I MHC incubated with peptides where the cell line-expressed MHC is not the expressed element *in vivo*. This provides a unique opportunity to proliferate $CD8^+$ cells which recognize a specific antigen associated with a particular MHC which would not be possible *in vivo* due to allelic restriction. For example, a peptide (NP) from the nuclear protein of Influenza virus is ordinarily restricted to the D^b molecule; however, we have found that such a peptide can bind to K^b (albeit more weakly than to D^b) and can generate a degree of thermal stability to the K^b (see Figure 3). Furthermore, K^b-expressing *Drosophila* cells preincubated with the NP peptide and cocultured with splenocytes from a B6 mouse results in the *in vitro* activation of $CD8^+$ which specifically recognize the K^b molecule associated with the NP peptide. In addition, the reciprocal experiment using a K^b-restricted peptide (OVA) derived from ovalbumin and D^b-expressing *Drosophila* cells results in the proliferation of $CD8^+$ which specifically recognize D^b containing the OVA peptide. Such CD8s are able to kill

cells (EL4 OVA) transfected with cDNA encoding the ovalbumin protein, indicating that *in vivo*, some D^b molecules are loaded with the OVA peptide.

This system therefore provides a unique opportunity to proliferate $CD8^+$ against specific antigens presented by a Class I molecule which, *in vivo*, is not the restriction element for that peptide. Although enough antigen is presented *in vivo* by said Class I for the cell to be recognized by $CD8^+$ and killed, it is not enough to proliferate such CD8s *in vivo*. By loading empty Class I molecules expressed by *Drosophila* cells with peptide, we are able to override the *in vivo* restriction by providing an excess of antigenic peptide to the Class I molecule in a non-competitive environment such that enough antigen is presented by the Class I to activate the specific $CD8^+$ recognizing this complex.

C. AIDS Treatment

In vitro activated cells may be administered to patients for *in vivo* therapy. Preferably, the Class I MHC genotype (haplotype) of the individual is first determined. Conventional tissue typing is appropriate for this purpose and may be performed at the treatment center or by some appropriate commercial operation. Once the individual's HLA type(s) is (are) determined, the best combination of peptides and Class I MHC molecules suitable for the individual patient is ascertained and prepared as noted above and the appropriate insect cell lines and peptides are provided. Resting or precursor $CD8^+$ T-cells from the blood of the patient are then stimulated with the appropriate peptide-loaded MHC produced by the insect cell culture. After activation, the $CD8^+$ cells are reintroduced into the patient's bloodstream, and the disease process in the patient continues to be monitored. Methods of removing and re-introducing cellular components are known in the art

and include procedures such as those exemplified in U.S. Patent No. 4,844,893 to Honsik, et al. and U.S. Patent No. 4,690,915 to Rosenberg.

Additional treatments may be administered as necessary until the disease is sufficiently remediated. Similar treatment protocols are appropriate for use with other immunosuppressed individuals, including transplant patients, elderly patients, and the like.

D. Cancer Treatment

In cancer patients, a treatment procedure similar to that described above is utilized. However, in such patients, it should be anticipated that conventional therapy to reduce the tumor mass may precede the immune therapy described herein. Therefore, it is preferred that blood samples from the putative patient be obtained and stored (e.g. via freezing) prior to the commencement of conventional therapy such as radiation or chemotherapy, which tends to destroy immune cells. Since few, if any, forms of cancer arise in direct response to viral infection, target peptides for immune treatment are less readily observed. However, recent studies indicate that mutations in the oncogenes *ras*, *neu*, and p53 contribute to cancer in as much as 50% of all cancer cases. Thus, peptides derived from these mutated regions of the molecules are prime candidates as targets for the present therapy. Pursuant to the protocols disclosed herein, the best combination of peptides and Class I molecules for the individual patient may be determined and administered.

For example, many tumors express antigens that are recognized *in vitro* by $CD8^+$ cells derived from the affected individual. Such antigens which are not expressed in normal cells may thus be identified, as well as the HLA type that presents them to the $CD8^+$ cells, for precisely targeted immunotherapy using the methods of the

present invention. For example, van der Bruggen, et al. have described an antigen whose expression is directed by a specific gene and which antigen appears to be presented by HLA A1 (Science 254: 1643-1647 (1991)). As various human tumor antigens are isolated and described, they become good candidates for immunotherapeutic applications as described herein.

In another, alternative therapeutic mode, it may be feasible to administer the *in vitro* activated $CD8^+$ cells of the present invention in conjunction with other immunogens. For example, the Large Multivalent Immunogen disclosed in U.S. Patent No. 5,045,320 may be administered in conjunction with activated $CD8^+$ cells.

It is also possible that cytokines such as IL-2 and IL-4, which mediate differentiation and activation of T-cells, may be administered as well, as cytokines are able to stimulate the T-cell response against tumor cells *in vivo*. It is believed that IL-2 plays a major role in the growth and differentiation of $CD8^+$ precursors and in $CD8^+$ proliferation. The administration of IL-2 to cancer patients is frequently associated with an improved anti-tumor response which is likely related to induction of tumor-specific T-cells. However, the best therapeutic effects of IL-2 might be obtained by continuous local rather than systemic administration of IL-2, thus minimizing the IL-2 toxicity and prolonging its biological activity. One may achieve local delivery via transfecting tumor cells with an IL-2 gene construct.

IL-2 cDNA is constructed as described by Karasuyama and Melchers in Eur. J. Immunol. 18: 97-104 (1988). The complete cDNA sequence of IL-2 is obtained as an Xho I fragment from the plasmid pBMGneo IL-2 (see Karasuyama and Melchers, *supra*) and directly ligated into the Sal I site in pRmHa-3. Recombinant pRmHa-3 plasmid with the insert in the correct orientation (determined

via restriction mapping with Hind III) is purified by cesium gradients and used to cotransfect S2 cells using the calcium phosphate technique. (A mixture of plasmid DNA was prepared for this purpose: 10μg pRmHa-3 containing IL-2 cDNA, 6μg each of pRmHa-3 plasmid containing MHC Class I heavy chain or β-2 microglobulin and 2μg of phshsneo DNA.) Stable cell lines which are inducible via $CuSO_4$ to express heavy chain, β-2 microglobulin and IL-2 were obtained by growing the transfectants in G418 medium. These stable cell lines were coated with peptide and used in the *in vitro* assay as described above. Tumor cells transfected with IL-2 are observed to enhance the CTL (CD8) activity against the parental tumor cells and bypass CD4 and T helper function in the induction of an antitumor or cytotoxic response *in vivo*. Therefore, increasing the potential of the *Drosophila* system via cotransfection with the IL-2 gene is suggested herein.

Example 9

Dose Dependence of the Production of Activated T-Cells Using the Antigen-Presenting System

Antigen-presenting cells (APC) were produced by transfecting *Drosophila* cells as described in Example 3 and then tested for their capacity to present antigen to T-cells from the 2C line of transgenic mice. With mouse cells as antigen-presenting cells, this line displays strong alloreactivity for L^d molecules complexed with an endogenous 8-mer peptide, p2Ca (Leu-Ser-Pro-Phe-Pro-Phe-Asp-Leu, SEQ ID NO 46), derived from a Krebs cycle enzyme, 2-oxoglutarate dehydrogenase (OGDH). The p2Ca peptide is exposed naturally bound to L^d on the surface of $H\text{-}2^d$ cells such as B10.D2 cells. The p2Ca peptide has intermediate binding affinity for soluble L^d molecules ($4 \times 10^6\ M^{-1}$) and high affinity for 2C TCR molecules (2×10^6 M^{-1} to $1 \times 10^7\ M^{-1}$).

A closely-related 9-mer peptide, QL9 (Gln-Leu-Ser-Pro-Phe-Pro-Phe-Asp-Leu, SEQ ID NO 47), has even higher affinity for these molecules (2×10^8 M^{-1} for L^d and 2×10^7 M^{-1} for 2C TCR). Except for one additional amino acid (glutamine) at the N terminus, QL9 has an identical sequence to p2Ca and, like p2Ca, forms part of the native sequence of OGDH.

With p2Ca and QL9 peptides (prepared in synthetic form), antigen-presenting cell requirements for mature unprimed 2C $CD8^+$ cells were studied *in vitro*. The responder $CD8^+$ cells were purified from pooled lymph nodes (LN) of 2C mice on a C57BL/6 (B6, H-2^b) background by first removing $CD4^+$ cells, class II-positive cells and B cells by mAb + complement treatment followed by positive panning.

Cell suspensions were prepared from pooled cervical, axillary, inguinal and mesenteric LN of young adult mice using a tissue grinder. For cell purification, LN cells were first treated with a cocktail of mAbs (anti-CD4, anti-HSA, anti-I-A^b) plus complement for 45 minutes at 37 °C. The surviving cells were further separated into $CD8^+$ and $CD8^-$ ($CD4^-$) cells by panning at 4 °C for 60-90 minutes on petri dishes coated with anti-CD8 mAb. The attached $CD8^+$ cells were recovered by incubation at 37 °C for 5 minutes followed by vigorous pipetting. Non-attached cells were eluted and treated with anti-CD8 mAb and complement to obtain $CD8^-$ $1B2^+$ 2C cells. More than 95% of the $CD8^+$ cells obtained were clonotype-positive ($IB2^+$) and 98% of these cells displayed a naive ($CD44^{lo}$) phenotype.

TCR stimulation elicited a complex pattern of intracellular events which lead to early up-regulation of CD69 and CD25 (IL-2 receptor or IL-2R) on the cell surface. These changes are apparent within a few hours of stimulation and are followed by cytokine synthesis and

cell proliferation. *Drosophila* cells were transfected with genes for L^d, L^d and B7.1 (L^d.B7), L^d and ICAM-1 (L^d.ICAM) or L^d, B7.1 and ICAM-1 (L^d.B7.ICAM). Figure 11 shows CD69 and CD25 expression on purified naive $CD8^+$ 2C cells stimulated for 12 hours with transfected *Drosophila* cells and p2Ca versus QL9 peptide at a concentration of 10 μM. Purified $CD8^+$ 2C cells were incubated with various *Drosophila* cells plus a peptide (either p2Ca or QL9, 10 μM) in bulk (2 ml) culture for 12 hours and then stained for CD69 or CD25. Either p2Ca or QL9 presented by *Drosophila* cells transfected with L^d.B7, L^d.ICAM or L^d.B7.ICAM were effective in stimulating the up-regulation of CD69 and CD25. However, non cultured 2C cells without either peptide or *Drosophila* cells did not show up-regulation of CD69 and CD25 (top panel).

In the presence of *Drosophila* cells expressing L^d alone, induction of CD69 and CD25 expression on $CD8^+$ 2C cells was low, but significant, with the strong QL9 peptide but barely detectable with the weaker p2Ca peptide. With L^d.B7 or L^d.ICAM antigen-presenting cells, both peptides elicited marked up-regulation of CD69 and CD25. However, L^d.B7.ICAM antigen-presenting cells induced even higher expression of these molecules.

Drosophila cells transfected with L^d alone failed to cause proliferation of 2C $CD8^+$ cells to either p2Ca or QL9 peptide in the absence of exogenous lymphokines, consistent with the minimal capacity of these cells as antigen-presenting cells to induce CD69 and CD25 expression. The results are shown in Figure 12. Responses to p2Ca (above) and QL9 (below) were measured by culturing 5×10^4 purified $CD8^+$ 2C cells with 2×10^5 *Drosophila* cells in the presence or absence of the indicated concentrations of peptides for 3 days. [^{3}H] TdR was added during the last 8 hours of culture; rIL-2 was added at a final concentration of 20 units/ml. The

data are the mean of triplicate cultures. When supplemented with exogenous IL-2, however, both peptides stimulated significant proliferative responses at high doses (10 μM). The proliferative responses elicited by QL9 were far stronger than for p2Ca (note the large difference between the scales of x-axes of the upper and the lower panel).

Dose-response relationships for proliferation on day 3 elicited by *Drosophila* cells transfected with L^d.B7.ICAM and presenting p2Ca peptide were compared to those elicited by *Drosophila* cells transfected with L^d.B7, L^d.ICAM or L^d.B7.ICAM presenting QL9 peptide. The results (means of triplicate cultures) are shown in Figure 13. *Drosophila* APC (2×10^5 cells) were cultured with 5×10^4 $CD8^+$ 2C cells for three days in the presence or absence of peptides. [^{3}H] TdR was added during the last 8 hours of culture; no IL-2 was added to the cultures.

Optimal proliferative responses elicited by L^d.B7.ICAM antigen-presenting cells on day 3 required high concentrations of p2Ca peptide, e.g. 10 μM (10^{-5} M), (Figure 13). The results for QL9 peptide were different. The does-response curves for L^d.B7 antigen-presenting cells plus QL9 and L^d.ICAM antigen-presenting cells plus QL9 approximated the results for L^d.B7.ICAM antigen-presenting cells plus p2Ca (Figure 13). However, with L^d.B7.ICAM antigen-presenting cells, proliferative responses to QL9 on day 3 were maximal with 100 nM (10^{-7} M) and were clearly apparent with doses as low as 10 pM (10^{-11} M) (Figure 13). At high doses, e.g. 10 μM (10^{-5} M), QL9 inhibited the proliferative response on day 3 (Figure 13, note the log scale).

Inhibition of proliferation by L^d.B7.ICAM antigen-presenting cells and QL9 peptide did not apply to IL-2 production (Figure 14, right), and was only seen

with high doses of antigen-presenting cells (Figure 14, left). L^d.B7.ICAM antigen-presenting cells plus QL9 peptide was again found to be more effective in eliciting IL-2 production than L^d.B7.ICAM antigen-presenting cells plus p2Ca peptide (Figure 14, right). The data indicates that antigen-presenting cells without peptides were ineffective in eliciting either response over the 100-fold range of antigen-presenting cells density tested (Figure 14, "-pep," open squares).

Changes in the dose-response relationships of the responses of $CD8^+$ 2C cells on Day 3, Day 4 and Day 5 were examined using QL9 peptide with L^d.B7, L^d.ICAM or L^d.B7.ICAM antigen-presenting cells. The results are shown in Figure 15. Responses were measured with 5×10^4 $CD8^+$ 2C cells and 3×10^5 antigen-presenting cells at the indicated peptide concentrations. The data are the mean results of triplicate cultures.

With an intermediate dose of 100 nM (10^{-7} M) QL9 peptide, proliferative responses to L^d.B7.ICAM antigen-presenting cells were high on Day 3 (Figure 15, left), reached a peak on Day 4 (Figure 15, center) and then declined to low levels on Day 5 (Figure 15, right). With a high dose of 10 μM (10^{-5} M) QL9 peptide, however, the response was low on Day 3 (Figure 15, left), but then increased markedly to reach a high peak on Day 5 (Figure 15, right).

Transient inhibition of proliferation induced by QL9 on Day 3 was only seen when the avidity of T-cell/APC interaction was very high. Decreasing the avidity of T-cell/APC interaction by using lower doses of APC (Figure 14, left) or lower doses of QL9 peptide (Figure 15, left) augmented the Day 3 proliferative response. Reducing the avidity of T-cell/APC interaction enhanced the early (Day 3) proliferative response but reduced the late (Day 5) response and also reduced IL-2

production (Figure 16, right). In Figure 16 the results obtained using $CD8^+$ 2C and $CD8^-$ 2C cells on Days 2, 3, 4 and 5 are compared.

The observations apply with the highly immunogenic L^d.B7.ICAM antigen-presenting cells. However, with the less immunogenic L^d.B7 or L^d.ICAM antigen-presenting cells, proliferative responses to QL9 peptide required high doses of peptide (Figure 13, Figure 15) and were crucially dependent upon CD8 expression by the responder cells (Figure 16). These responses with L^d.B7 and L^d.ICAM antigen-presenting cells reached a peak on Days 3 or 4 (rather than Day 5) and were far lower than with L^d.B7.ICAM antigen-presenting cells. With low doses of QL9, e.g. 1 nM (10^{-9} M), proliferative responses with L^d.B7 and L^d.ICAM antigen-presenting cells were completely undetectable (< 100 cpm) (Figure 13). This was in striking contrast to the results seen using L^d.B7.ICAM antigen-presenting cells, where 1 nM QL9 led to high responses (> 10,000 cpm) (Figure 13). In contrast to the results with high doses of QL9 peptide (10 μM, Table 2), the synergistic interaction between B7 and ICAM for proliferative responses became pronounced at low peptide doses.

As can be seen, L^d.B7.ICAM cells act as extremely potent antigen-presenting cells for naive 2C cells. Raising the avidity of T-cell/APC interaction to a high level inhibits the early proliferative response but potentiates the late response and IL-2 production is enhanced. For the high-affinity peptides such as QL9, the synergy between B7 and ICAM is pronounced at low doses of antigen.

Example 10
Production of Cytotoxic T-Cells Using the Antigen Presenting System

Antigen-presenting cells (APC) were produced by transfecting *Drosophila* cells as described in Example 3 and then tested for their ability to induce CTL activity. CTL activity was tested on ^{51}Cr-labelled RMA.S-L^d targets sensitized with QL9 peptide, no peptide or an irrelevant peptide (MCMV). $CD8^+$ 2C cells (5×10^5) were cultured with 2×10^6 transfected *Drosophila* cells in a volume of 2 ml in a 24-well culture plate. Cai, Z., and J. Sprent, (1994). Peptides were present during the culture at a concentration of 10 mM. After 4 days, the cells were pooled and adjusted to the required number. To prepare targets, RMA-S.L^d cells were labeled with ^{51}Cr (100 mCi/1-2 x 10^6 cells) at 37 °C for 90 minutes in the presence or absence of peptides. After labeling, the cells were thoroughly washed and resuspended in medium with or without peptides. Specific ^{51}Cr release was calculated according to established procedure. Id.

The capacity of L^d.B7, L^d.B7.ICAM and L^d.ICAM antigen-presenting cells to induce CTL activity to 10 μM QL9 peptide in bulk cultures is shown in Figure 17. Strongly immunogenic L^d.B7.ICAM antigen-presenting cells were efficient in generating QL9-specific CTL (Figure 17, center). Significantly, L^d.B7 antigen-presenting cells were also effective in generating CTL to QL9 (Figure 17, left). The surprising finding, however, was that L^d.ICAM antigen-presenting cells were totally unable to stimulate CTL generation (Figure 17, right). This result (representative of three different experiments) was unexpected, because L^d.ICAM antigen-presenting cells were no less efficient than L^d.B7 antigen-presenting cells at inducing proliferative responses to QL9 (Figures 13 and 15). The L^d.ICAM-stimulated cultures in Figure 17

contained large numbers of blast cells, and total cell yields were about 3-fold higher than the input number.

The surprising finding that L^d.ICAM antigen-presenting cells were totally unable to stimulate CTL generation applied to cultures not supplemented with exogenous lymphokines. However, when exogenous IL-2 was added to the cultures, L^d.ICAM antigen-presenting cells induced strong CTL activity to QL9 (Figure 18, right; 20u/ml exogenous IL-2).

Example 11
Proliferation of Normal T-Cells Induced by the Antigen-Presenting System

Antigen-presenting cells (APC) were produced by transfecting *Drosophila* cells as described in Example 3 and then tested for their ability to induce proliferation in normal (nontransgenic) murine T-cells.

The capacity of L^d.B7.ICAM *Drosophila* cells to induce a strong primary response of 2C TCR transgenic $CD8^+$ cells raised the question whether *Drosophila* cells could act as antigen-presenting cells for normal (nontransgenic) $CD8^+$ cells. Since the 2C mice were on a B6 ($H\text{-}2^b$) background, the response of normal B6 $CD8^+$ cells was tested. Graded doses of $CD8^+$ cells from normal B6 mice were cultured with 10 μM QL9 peptide presented by L^d.B7.ICAM Drosophila antigen-presenting cells, i.e. a situation where a diverse repertoire of T-cells was exposed to only a single alloantigen (L^d + QL9) but at high concentration. As shown in Figure 19 (left), presentation of QL9 by L^d.B7.ICAM antigen-presenting cells was indeed immunogenic for normal B6 $CD8^+$ cells and led to appreciable proliferative responses on Day 3 (80,000 cpm) with large doses of responder cells (1×10^6) in the absence of added cytokines; responses to an unrelated peptide, MCMV, were much lower (though

significant) and no response occurred in the absence of peptide. As expected, the response of normal B6 $CD8^+$ cells to QL9 plus L^d.B7.ICAM antigen-presenting cells was substantially lower than with normal B10.D2 spleen cells as antigen-presenting cells (where the allostimulus was provided by a multiplicity of self peptides bound to L^d, K^d and D^d, but at low concentration) (Figure 19, right. Note the difference in the Y axis scales).

Example 12
Activation Of Cytotoxic T-Cells Using Immobilized Purified Recombinant MHC Class I Molecules And Assisting Molecules

Except as noted, cells, materials and reagents were prepared as described in Example 4. Biotinylated anti-mouse CD28 monoclonal antibody (clone 37.51) was purchased from Pharmingen (San Diego, CA). This antibody is also available from Caltag (South San Francisco, CA). This antibody, which binds to CD28 co-stimulatory receptor, a ligand of B7.1 and B7.2 on the surface of T-cells, augments the proliferation of T-cells (Gross et al. J. Immunol. 149, 380-388, 1992). IL-2 was used as a concanavalin A supernatant (10% final concentration).

Substrates were prepared as follows: Fifty microliters of PBS containing 1 microgram/ml of neutravidin were added to each well of a 96 well cell culture plate (Corning cat.#25860). After 2 hours at room temperature, the wells were washed 3 times with PBS prior to incubation with biotinylated molecules. Avidin-coated mouse red blood cells were prepared as described for avidin-coated sheep red blood cells in Example 4. Avidin-coated latex beads were prepared as described Example 4.

Biotinylation of recombinant L^d was performed as described in Example 4. Biotinylated recombinant L^d

was immobilized on the substrate together with biotinylated anti-CD28 antibody. Avidin-coated red blood cells or latex beads were incubated with 0.2 mg/ml biotinylated L^d, 0.025 mg/ml biotinylated anti-CD28, or a mixture thereof for 30 minutes at room temperature, then washed 3 times in DMEM containing 10% FCS and used immediately. Avidin-coated 96 well plates were incubated with 50 microliters per well of 2 microgram/ml biotinylated L^d, 0.25 microgram/ml anti-CD28, or mixtures thereof for 30 minutes at room temperature, then washed 3 times using DMEM containing 10% FCS and used immediately.

2C+ T-cells were prepared as described in Example 4. $CD8^+$ cells from C57BL/6 mice were prepared from the lymph nodes of these mice by magnetic depletion. Purified cells were consistently 90-92% positive for CD8 expression, as assessed by flow cytofluorometry.

T-cell activation was performed in culture plates coated with purified L^d molecules and anti-CD28 antibody. T-cells and peptide (0.02 mM final concentration) were added to 96 well plates coated with L^d and/or anti-CD28, in a final volume of 0.2ml/well and cultured for the appropriate time at 37 °C in humid atmosphere containing 5% CO_2.

T-cell activation using red blood cells or latex beads coated with purified L^d molecules and anti-CD28 antibody was performed in uncoated culture plates. T-cells and peptide (0.02mM) were added to each well, together with 100,000 red blood cells or latex beads. Final volume was 0.2ml. Culture conditions were as described above.

T-cell mitogenesis was assayed by incorporation of a pulse of 1 μCi of tritiated thymidine per well by triplicate cell cultures. Cells were harvested 8 hours later and thymidine incorporation was determined by counting the filters in a scintillation counter.

RMA.S cells (target cells) expressing L^d were incubated with radiolabeled chromium for 90 minutes at 37 °C, washed 3 times and distributed in 96 well U-bottom plate (Costar cat#3799) (5000-10000 cells per well) in the presence of the appropriate peptide at 0.01mM. Various amounts of activated T-cells (effector cells) were added to reach effector/target ratios (E/T ratios) ranging between 150 and 1. After 5 hours of incubation at 37 °C, 0.1ml of supernatant was collected from each well and counted in a gamma counter. Percent lysis was determined by a standard method (Coligan et al., Current Protocols in Immunology, section 3.11, Wiley, New York (1991)).

Immobilized purified L^d and anti-CD28 antibody were mitogenic for 2C+ T-cells. When QL9 peptide was used, a thymidine incorporation above 100,000 cpm per well was consistently measured by day 3-5 of culture (Figure 20). Maximum thymidine incorporation in that same range was obtained using any of the three methods of activation (molecules immobilized on plastic, on red blood cells or on latex beads). When activation molecules were immobilized on plastic, L^d alone immobilized on plastic induced a transient mitogenesis (Figure 20, broken line) whereas L^d plus anti-CD28 antibody induced a higher and more sustained mitogenesis (Figure 20, solid line). Anti-CD28 antibody in addition to L^d was required for the induction of any mitogenesis in the model using red blood cells. However, anti-CD28 antibody alone did not induce any mitogenesis.

QL9-L^d complexes are recognized by the 2C T-cell receptor with a high affinity. Complexes of other peptides and L^d recognized by this receptor with a lower affinity were able to activate 2C+ T-cells; these included peptides p2Ca and SL9 (Figure 21). However, IL-2 added at day 2 of culture was required in order to

observe mitogenesis using these peptides. Activation was peptide specific since LCMV peptide, a control peptide that is not recognized by the 2C T-cell receptor, did not induce activation. Mitogenesis was measurable starting with as little as 700 T-cells per well (96 plate well), demonstrating that the method activated a very small number of T-cells in a peptide specific manner.

2C+ T-cells were mixed with total $CD8^-$ cells from C57BL/6 mice at a 1/99 ratio and cells were cultured with immobilized L^d and anti-CD28 antibody in the presence of peptide QL9. IL-2 was added at day 2 of culture, and subsequently every other day. Cell activation and proliferation was noted, as evidenced by the formation of clumps, presence of enlarged cells that progressively spread in the well. At day 12 of culture, cells were harvested and percentage of cells expressing the 2C T-cell receptor was assessed by staining with the anticlonotypic antibody 1B2 and analysis by flow cytofluorometry: 43% of the cells cultured initially in the presence of peptide QL9 were 2C+, (Figure 22) whereas 49% of the cells expressed 2C after stimulation with peptide p2Ca (Figure 23), and 22% with peptide SL9 (Figure 24). This shows a considerable enrichment (43, 49 and 22 times) in specific T-cells after 12 days of culture. Enrichment was observed even with SL9, a peptide that makes a low affinity complex with the 2C T-cell receptor. This method thus can specifically activate and enrich a small subpopulation of antigen specific T-cells out of a heterogenous mixture of T-cells.

2C+ T-cells, activated using L^d and anti-CD28 antibody in the presence of QL9 peptide, were cultured for 5 days, then their cytotoxic capacity was assessed. The results, shown in Figure 25, demonstrated that immobilized activation molecules induced the cells to

differentiate into effector cytotoxic T-cells. Lysis was specific, since it was observed in the presence of QL9 peptide but not in the presence of a control peptide (LCMV). Resting T-cells were thus activated using immobilized molecules to differentiate into cytotoxic T-cells able to specifically kill targets.

As can be seen, immobilized purified MHC Class I and assisting molecules can be used to specifically activate naive resting T-cells into cytotoxic T-cells. MHC class I and assisting molecules are sufficient for activation; no additional signal originating from antigen-presenting cells is necessary. MHC class I and assisting molecules immobilized on substrates, such as cell culture plates, provide an appropriate tool for T-cell activation. Several advantages are offered by such coated substrates. They are easy to prepare and to manipulate, they can be coated with well-controlled amounts of molecules, ensuring reproducible activation conditions.

Example 13

Activation of Human Cytotoxic T-Cells

A unit of human blood (450 mls) collected in heparin (10 μ/ml) was obtained through the General Clinical Research Center (GCRC) at Scripps Clinic, La Jolla, CA. The blood was first processed in a Ficoll-Hypaque density gradient in a 50 cc conical centrifuge (Histopaque 1077, Sigma) according to the manufacturer's specifications. Once the buffy coat was obtained, the sample was washed in buffer 1 (D-PBS without Ca^{++} or Mg^{++}), then buffer 2 (RPMI with 4% fetal calf serum (FCS)) and a final wash in buffer 3 (D-PBS + 1% human serum albumin (HSA, 25% Buminate/Baxter-Hyland) and 0.2% sodium citrate (w/v)).

The total peripheral blood mononuclear cell (PBMCs) preparation was counted from the washed cells. This preparation was then taken through a MaxSep™ isolation procedure (Baxter) where $CD8^-$ cells were selected by negative selection. A cocktail of mAbs to cells targeted for removal (CD19-PharMingen, CD4-Ortho-mune, CD15-PharMingen, CD56-PharMingen, CD14-PRI) was prepared at 2 µg/ml of cells. The total PBMC cell count was diluted to 20 x 10^6/ml in buffer 3 and the antibodies were added.

The mixture (approximately 40 mls) was rotated (4 rpm) on a rotary shaker at 4 °C, so that the tube mixed the sample end over end, for a total of 30 minutes. After the sensitization phase, the cells were washed with buffer 3 and resuspended in the same buffer with magnetic Dynal beads coated with sheep-anti-mouse IgG (SAM) (Dynabeads M450 #110.02). The stock of beads is usually 4 x 10^8 beads/ml. The final bead:target cell ratio is 10:1.

To determine the final volume of beads to use, the formulas below were followed:

(Total cells sensitized) x (% of population of target cells) = Total theoretical target cell number.

Total theoretical target cell number x 10 = Total beads required.

Total beads required/Bead concentration of stock = Volume of beads required.

The volume of the final sensitized cells:beads mixture was approximately 50 mls. The mixture was put into a 150 ml Fenwal Transfer Pack (#4R2001), air added with a needle and the mixture rotating under similar conditions as above (30 minutes, 4 °C, 4 rpm, agitated end-over-end). At the end of the incubation period, the target cells were removed with a MaxSep™ separation device according to the manufacture's specifications.

The separated cells were transferred from the bag and counted to determine recovery. FACS analysis was performed to determine the purity of the sample.

The resulting separated human $CD8^+$ cells were stimulated with *Drosophila* (fly) antigen-presenting cells which were transfected and shown to express human HLA A2.1, B7.1 and/or LFA-3. The fly cells were diluted to 10^6/ml in Schneider's medium with 10% FCS serum. On the following day CuSO4 was added to the cultured cells which were incubated for 24 hours at 27 °C and harvested. The harvested cells were washed and suspended in Insect X-press medium with a final peptide concentration of 100 μg/ml and incubated for 3 hours at 27 °C.

The peptides used were HIV-RT (ILKEPVHGV) (SEQ ID NO 48), Tyrosinase (YMNGTMSQV) (SEQ ID NO 49), and Influenza Matrix (GILGFVFTL) (SEQ ID NO 50).

The control peptide was derived from the core of the hepatitis virus and had the sequence FLPSDFFPSV (SEQ ID NO 51).

The fly antigen-presenting cells were added to the $CD8^+$ cells at a ratio of 1:10 (APC:$CD8^+$ T-cell). The cells were incubated in flat bottom wells (48 wells) for four days at 37 °C. At day 5, IL-2 (10 μ/ml) and IL-7 (10 μ/ml) (Genzyme) were added with a media change. On day 11 a CTL assay was performed.

JY, an EBV-transformed human B cell line expressing HLA 2.1, B7 was used as the target cell in the chromium release assay. Vissereu, M.J.W. et al. J. Immunol., 154:3991-3998 (1995). JY cells were seeded at 3×10^5 cells/ml in RPMI with 10% FCS, 24 hours prior to the assay. The JY cells were counted and washed once in RPMI wash solution (4% Rehautin FCS, 1% HEPES, 0.025% gentamycin in RPMI) to provide 5×10^6 cells per sample. The cell pellet was gently resuspended in 100 μl of ^{51} Chromium stock (0.1 mCi, NEN) and placed in a 37 °C water

bath for 1 hour with agitation every fifteen minutes. Labelled JY cells were washed four times for 6 minutes each at 1400 rpm in 10 mls of RPMI wash solution. Cells were adjusted to 1 x 10^5 cells/ml in RPMI/10% Hyclone. Efficiency of target cell labelling is confirmed by standard gamma counter techniques.

For peptide loading of target cells, two mls of labelled JY cells (2 x 10^5 cells) were incubated with 10 µg/ml peptide for 30 minutes at room temperature. Peptide stock solutions (1mg/ml) were stored at -70 °C. In a 96 well round bottom plate, 100 µl effector cells and 100 µl peptide loaded target cells were combined at ratios of 84:1, 17:1 and 3.4:1 (effector:target). Controls to measure spontaneous release and maximum release of ^{51}Cr were included in duplicate. Samples were incubated at 37 °C for 6 hours.

K562 cells at a concentration of 10^7/ml in RPMI with 10% FCS were added at a ratio of 20:1 (unlabelled K562:labelled JY). This erythroleukemic cell line was used to reduce NK background cell lysis in the chromium release assay. Plates were centrifuged at 1000 rpm for 5 minutes and 100 µl supernatant from each sample transferred to 96 tube collection tubes. Analysis of cell lysis is determined by standard gamma counting techniques (Gammacell 1000, Nordion).

CTL activity produced by activating human $CD8^+$ T-cells with antigen-presenting cells loaded with influenza matrix peptide (SEQ ID NO 50) is shown in Figure 26. Prior exposure to influenza virus indicates that this CTL activity was a secondary response. Data points are the mean of values from triplicate cultures. Antigen-presenting cells expressing A2.1, B7.1 and ICAM-1 were more effective than antigen-presenting cells expressing A2.1 and B7.1 or antigen-presenting cells expressing A2.1, B7.1 and LFA-3.

The results of activating human CD8+ T-cells with antigen-presenting cells loaded with HIV-RT peptide (SEQ ID NO 48) are presented in Figure 27. Since screening of the blood of this patient had indicated no prior exposure to HIV, these results indicate the CTL activity was based on a primary response. In this case, only antigen-presenting cells expressing A2.1, B7.1 and ICAM-1 produced CTL activity that was significantly greater than control levels.

Figure 28 shows the CTL activity of human CD8+ T-cells activated by antigen-presenting cells loaded with tyrosinase peptide (SEQ ID NO 49). Tyrosinase is a normally occurring enzyme that is over-expressed in melanoma tumor cells. Again, the antigen-presenting cells expressing all three molecules, ICAM-1 as well as A2.1 and B7.1, were most effective, especially at the lowest effector:target ratio tested.

These results show that the antigen presenting system is capable of producing effective CTL activity in human CD8+ T-cells directed against a peptide that is derived from an endogenous protein that is over-expressed in tumor cells. This also demonstrates that this *in vitro* stimulation of CD8+ T-cells using both B7 and ICAM will generate cytotoxic CD8+ T-cells even against peptides that would otherwise be recognized as self. This is contrary to present knowledge that such "self" peptides could not be used to create cytotoxic T-cells. This method greatly expands the number of possible tumor specific antigens that can be used to activate CD8+ T-cells against the tumor.

The foregoing is intended to be illustrative of the present invention, but not limiting. Numerous variations and modifications may be effected without departing from the true spirit and scope of the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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Brunmark, Anders
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(ii) TITLE OF INVENTION: ANTIGEN PRESENTING SYSTEM AND METHODS FOR ACTIVATION OF T-CELLS

(iii) NUMBER OF SEQUENCES: 51

(iv) CORRESPONDENCE ADDRESS:
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(C) CITY: Chicago
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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 8-MAR-1996
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/400,338
(B) FILING DATE: 8-MAR-1995
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(B) REGISTRATION NUMBER: 30,203
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(ix) TELECOMMUNICATION INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCACCATGGC CGTCATGGCG CCC
23

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGTCACACTT TACAAGCTCT GAG
23

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCACCATGCT GGTCATGGCG CCC
23

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGACTCGATG TGAGAGACAC ATC
23

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCACCATGCG GGTCATGGCG CCC
23

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGTCAGGCTT TACAAGCGAT GAG
23

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCACCATGCG GGTAGATGCC CTC
23

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGTTACAAGC TGTGAGACTC AGA
23

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCACCATGGC GCCCCGAAGC CTC
23

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GGTCACACTT TATTAGCTGT GAG
23

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCACCATGGC GCCCCGAACC CTC
23

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGTCACAATT TACAAGCCGA GAG
23

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 427 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AATTCGTTGC AGGACAGGAT GTGGTGCCCG ATGTGACTAG CTCTTTGCTG CAGGCCGTCC
60

TATCCTCTGG TTCCGATAAG AGACCCAGAA CTCCGGCCCC CCACCGCCCA CCGCCACCCC
120

CATACATATG TGGTACGCAA GTAAGAGTGC CTGCGCATGC CCCATGTGCC CCACCAAGAG
180

TTTTGCATCC CATACAAGTC CCCAAAGTGG AGAACCGAAC CAATTCTTCG CGGGCAGAAC
240

AAAAGCTTCT GCACACGTCT CCACTCGAAT TTGGAGCCGG CCGGCGTGTG CAAAAGAGGT
300

GAATCGAACG AAAGACCCGT GTGTAAAGCC GCGTTTCCAA AATGTATAAA ACCGAGAGCA
360

TCTGGCCAAT GTGCATCAGT TGTGGTCAGC AGCAAAATCA AGTGAATCAT CTCAGTGCAA
420

CTAAAGG
427

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 740 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

ATTCGATGCA CACTCACATT CTTCTCCTAA TACGATAATA AAACTTTCCA TGAAAAATAT
60

GGAAAAATAT ATGAAAATTG AGAAATCCAA AAAACTGATA AACGCTCTAC TTAATTAAAA
120

TAGATAAATG GGAGCGGCTG GAATGGCGGA GCATGACCAA GTTCCTCCGC CAATCAGTCG
180

TAAAACAGAA GTCGTGGAAA GCGGATAGAA AGAATGTTCG ATTTGACGGG CAAGCATGTC
240

TGCTATGTGG CGGATTGCGG AGGAATTGCA CTGGAGACCA GCAAGGTTCT CATGACCAAG
300

AATATAGCGG TGTGAGTGAG CGGGAAGCTC GGTTTCTGTC CAGATCGAAC TCAAAACTAG
360

TCCAGCCAGT CGCTGTCGAA ACTAATTAAG TTAATGAGTT TTTCATGTTA GTTTCGCGCT
420

GAGCAACAAT TAAGTTTATG TTTCAGTTCG GCTTAGATTT CGCTGAAGGA CTTGCCACTT
480

TCAATCAATA CTTTAGAACA AAATCAAAAC TCATTCTAAT AGCTTGGTGT TCATCTTTTT
540

TTTTAATGAT AAGCATTTTG TCGTTTATAC TTTTTATATT TCGATATTAA ACCACCTATG
600

AAGTTCATTT TAATCGCCAG ATAAGCAATA TATTGTGTAA ATATTTGTAT TCTTTATCAG
660

GAAATTCAGG GAGACGGGGA AGTTACTATC TACTAAAAGC CAAACAATTT CTTACAGTTT
720

TACTCTCTCT ACTCTAGAGT
740

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GCTTGGATCC AGATCTACCA TGTCTCGCTC CGTGGCCTTA GCTGTGCTCG CGCTACTCTC
60

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GGATCCGGAT GGTTACATGT CGCGATCCCA CTTAAC
36

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GGAGCCGTGA CTGACTGAG
19

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CCCTCGGCAC TGACTGACTC CTAG
24

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GATCCTTATT AGATCTCACC ATCACCATCA CCATTGAG
38

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TCGACTCAAT GGTGATGGTG ATGGTGAGAT CTAATAAG
38

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3875 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TTGCAGGACA GGATGTGGTG CCCGATGTGA CTAGCTCTTT GCTGCAGGCC GTCCTATCCT 60

CTGGTTCCGA TAAGAGACCC AGAACTCCGG CCCCCCACCG CCCACCGCCA CCCCCATACA 120

TATGTGGTAC GCAAGTAAGA GTGCCTGCGC ATGCCCCATG TGCCCCACCA AGAGCTTTGC 180

ATCCCATACA AGTCCCCAAA GTGGAGAACC GAACCAATTC TTCGCGGGCA GAACAAAAGC 240

TTCTGCACAC GTCTCCACTC GAATTTGGAG CCGGCCGGCG TGTGCAAAAG AGGTGAATCG 300

AACGAAAGAC CCGTGTGTAA AGCCGCGTTT CCAAAATGTA TAAAACCGAG AGCATCTGGC 360

CAATGTGCAT CAGTTGTGGT CAGCAGCAAA ATCAAGTGAA TCATCTCAGT GCAACTAAAG 420

GGGAATTCGA GCTCGGTACC CGGGGATCCT TATTAGATCT CACCATCACC ATCACCATTG 480

AGTCGACCTG CAGGCATGCA AGCTATTCGA TGCACACTCA CATTCTTCTC CTAATACGAT 540

AATAAAACTT TCCATGAAAA ATATGGAAAA ATATATGAAA ATTGAGAAAT CCAAAAAACT 600

GATAAACGCT CTACTTAATT AAAATAGATA AATGGGAGCG GCAGGAATGG CGGAGCATGG 660

CCAAGTTCCT CCGCCAATCA GTCGTAAAAC AGAAGTCGTG GAAAGCGGAT AGAAAGAATG 720

TTCGATTTGA CGGGCAAGCA TGTCTGCTAT GTGGCGGATT GCGGAGGAAT TGCACTGGAG 780

ACCAGCAAGG TTCTCATGAC CAAGAATATA GCGGTGAGTG AGCGGGAAGC TCGGTTTCTG 840

TCCAGATCGA ACTCAAAACT AGTCCAGCCA GTCGCTGTCG AAACTAATTA AGTCAATGAG 900

TTTTTCATGT TAGTTTCGCG CTGAGCAACA ATTAAGTTTA TGTTTCAGTT CGGCTTAGAT 960

TTCGCTGAAG GACTTGCCAC TTTCAATCAA TACTTTAGAA CAAAATCAAA ACTCATTCTA 1020

ATAGCTTGGT GTTCATCTTT TTTTTTAATG ATAAGCATTT TGTCGTTTAT ACTTTTTATA 1080

TTTCGATATT AAACCACCTA TGAAGTCtAT TTTAATCGCC AGATAAGCAA TATATTGTGT 1140

AAATATTTGT ATTCTTTATC AGGAAATTCA GGGAGACGGG AAGTTACTAT CTACTAAAAG 1200

CCAAACAATT TCTTACAGTT TTACTCTCTC TACTCTAGAG TAGCTTGGCA CTGGCCGTCG 1260

TTTTACAACG TCGTGACTGG GAAAACCCTG GCGTTACCCA ACTTAATCGC CTTGCAGCAC 1320

ATCCCCCTTT CGCCAGCTGG CGTAATAGCG AAGAGGCCCG CACCGATCGC CCTTCCCAAC 1380

AGTTGCGCAG CCTGAATGGC GAATGGCGCC TGATGCGGTA TTTTCTCCTT ACGCATCTGT 1440

GCGGTATTTC ACACCGCATA TGGTGCACTC TCAGTACAAT CTGCTCTGAT GCCGCATAGT 1500

TAAGCCAGCC CCGACACCCG CCAACACCCG CTGACGCGCC CTGACGGGCT TGTCTGCTCC 1560

CGGCATCCGC TTACAGACAA GCTGTGACCG TCTCCGGGAG CTGCATGTGT CAGAGGTTTT 1620

CACCGTCATC ACCGAAACGC GCGAGACGAA AGGGCCTCGT GATACGCCTA TTTTTATAGG 1680

TTAATGTCAT GATAATAATG GTTTCTTAGA CGTCAGGTGG CACTTTTCGG GGAAATGTGC 1740

GCGGAACCCC TATTTGTTTA TTTTTCTAAA TACATTCAAA TATGTATCCG CTCATGAGAC 1800

AATAACCCTG ATAAATGCTT CAATAATATT GAAAAAGGAA GAGTATGAGT ATTCAACATT 1860

TCCGTGTCGC CCTTATTCCC TTTTTTGCGG CATTTTGCCT TCCTGTTTTT GCTCACCCAG 1920

AAACGCTGGT GAAAGTAAAA GATGCTGAAG ATCAGTTGGG TGCACGAGTG GGTTACATCG 1980

AACTGGATCT CAACAGCGGT AAGATCCTTG AGAGTTTTCG CCCCGAAGAA CGTTTTCCAA 2040

TGATGAGCAC TTTTAAAGTT CTGCTATGTG GCGCGGTATT ATCCCGTATT GACGCCGGGC 2100

AAGAGCAACT CGGTCGCCGC ATACACTATT CTCAGAATGA CTTGGTTGAG TACTCACCAG 2160

TCACAGAAAA GCATCTTACG GATGGCATGA CAGTAAGAGA ATTATGCAGT GCTGCCATAA 2220

CCATGAGTGA TAACACTGCG GCCAACTTAC TTCTGACAAC GATCGGAGGA CCGAAGGAGC 2280

TAACCGCTTT TTTGCACAAC ATGGGGGATC ATGTAACTCG CCTTGATCGT TGGGAACCGG 2340

AGCTGAATGA AGCCATACCA AACGACGAGC GTGACACCAC GATGCCTGTA GCAATGGCAA 2400

CAACGTTGCG CAAACTATTA ACTGGCGAAC TACTTACTCT AGCTTCCCGG CAACAATTAA 2460

TAGACTGGAT GGAGGCGGAT AAAGTTGCAG GACCACTTCT GCGCTCGGCC CTTCCGGCTG 2520

GCTGGTTTAT TGCTGATAAA TCTGGAGCCG GTGAGCGTGG GTCTCGCGGT ATCATTGCAG 2580

CACTGGGGCC AGATGGTAAG CCCTCCCGTA TCGTAGTTAT CTACACGACG GGGAGTCAGG 2640

CAACTATGGA TGAACGAAAT AGACAGATCG CTGAGATAGG TGCCTCACTG ATTAAGCATT 2700

GGTAACTGTC AGACCAAGTT TACTCATATA TACTTTAGAT TGATTTAAAA CTTCATTTTT 2760

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ATCCTTTTTT TCTGCGCGTA ATCTGCTGCT TGCAAACAAA AAAACCACCG CTACCAGCGG 2940

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GAGCGCAGAT ACCAAATACT GTCCTTCTAG TGTAGCCGTA GTTAGGCCAC CACTTCAAGA 3060

ACTCTGTAGC ACCGCCTACA TACCTCGCTC TGCTAATCCT GTTACCAGTG GCTGCTGCCA 3120

GTGGCGATAA GTCGTGTCTT ACCGGGTTGG ACTCAAGACG ATAGTTACCG GATAAGGCGC 3180

AGCGGTCGGG CTGAACGGGG GGTTCGTGCA CACAGCCCAG CTTGGAGCGA ACGACCTACA 3240

CCGAACTGAG ATACCTACAG CGTGAGCATT GAGAAAGCGC CACGCTTCCC GAAGGGAGAA 3300

AGGCGGACAG GTATCCGGTA AGCGGCAGGG TCGGAACAGG AGAGCGCACG AGGGAGCTTC 3360

CAGGGGGAAA CGCCTGGTAT CTTTATAGTC CTGTCGGGTT TCGCCACCTC TGACTTGAGC 3420

GTCGATTTTT GTGATGCTCG TCAGGGGGGC GGAGCCTATG GAAAAACGCC AGCAACGCGG 3480

CCTTTTTACG GTCCTGGCCT TTTGCTGGCC TTTTGCTCAC ATGTCTTTCC TGCGTTATCC 3540

CCTGATTCTG TGGATAACCG TATTACCGCC TTTGAGTGAG CTGATACCGC TCGCCGCAGC 3600

CGAACCGACC GAGCGCAGCG AGTCAGTGAG CGAGGAAGCG GAAGAGCGCC CAATACGCAA 3660

ACCGCCTCTC CCCGCGCGTT GGCCGATTCA TTAATGCAGC TGGCACGACA GGTTTCCCGA 3720

CTGGAAAGCG GGCAGTGAGC GCAACGCAAT TAATGTGAGT TAGCTCACTC ATTAGGCACC 3780

CCAGGCTTTA CACTTTATGC TTCCGGCTCG TATGTTGTGT GGAATTGTGA GCGGATAACA 3840

ATTTCACACA GGAAACAGCT ATGACATGAT TACCG 3875

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 71 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GATCCTTATT AGATCTTACC CATACGACGT CCCAGATTAC GCTCGATCTC ACCATCACCA 60

TCACCATTGA G 71

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 71 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TCGACTCAAT GGTGATGGTG ATGGTGAGAT CGAGCGTAAT CTGGGACGTC GTATGGGTAA 60

GATCTAATAA G 71

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3908 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TTGCAGGACA GGATGTGGTG CCCGATGTGA CTAGCTCTTT GCTGCAGGCC GTCCTATCCT 60

CTGGTTCCGA TAAGAGACCC AGAACTCCGG CCCCCCACCG CCCACCGCCA CCCCCATACA 120

TATGTGGTAC GCAAGTAAGA GTGCCTGCGC ATGCCCCATG TGCCCCACCA AGAGcTTTGC 180

ATCCCATACA AGTCCCCAAA GTGGAGAACC GAACCAATTC TTCGCGGGCA GAACAAAAGC 240

TTCTGCACAC GTCTCCACTC GAATTTGGAG CCGGCCGGCG TGTGCAAAAG AGGTGAATCG 300

AACGAAAGAC CCGTGTGTAA AGCCGCGTTT CCAAAATGTA TAAAACCGAG AGCATCTGGC 360

CAATGTGCAT CAGTTGTGGT CAGCAGCAAA ATCAAGTGAA TCATCTCAGT GCAACTAAAG 420

GGGAATTCGA GCTCGGTACC CGGGGATCCT TATTAGATCT TACCCATACG ACGTCCCAGA 480

TTACGCTCGA TCTCACCATC ACCATCACCA TTGAGTCGAC CTGCAGGCAT GCAAGCTATT 540

CGATGCACAC TCACATTCTT CTCCTAATAC GATAATAAAA CTTTCCATGA AAAATATGGA 600

AAAATATATG AAAATTGAGA AATCCAAAAA ACTGATAAAC GCTCTACTTA ATTAAAATAG 660

ATAAATGGGA GCGGCAGGAA TGGCGGAGCA TGGCCAAGTT CCTCCGCCAA TCAGTCGTAA 720

AACAGAAGTC GTGGAAAGCG GATAGAAAGA ATGTTCGATT TGACGGGCAA GCATGTCTGC 780

TATGTGGCGG ATTGCGGAGG AATTGCACTG GAGACCAGCA AGGTTCTCAT GACCAAGAAT 840

ATAGCGGTGA GTGAGCGGGA AGCTCGGTTT CTGTCCAGAT CGAACTCAAA ACTAGTCCAG 900

CCAGTCGCTG TCGAAACTAA TTAAGTtAAT GAGTTTTTCA TGTTAGTTTC GCGCTGAGCA 960

ACAATTAAGT TTATGTTTCA GTTCGGCTTA GATTTCGCTG AAGGACTTGC CACTTTCAAT 1020

CAATACTTTA GAACAAAATC AAAACTCATT CTAATAGCTT GGTGTTCATC TTTTTTTTTA 1080

ATGATAAGCA TTTTGTCGTT TATACTTTTT ATATTTCGAT ATTAAACCAC CTATGAAGTc 1140

tATTTTAATC GCCAGATAAG CAATATATTG TGTAAATATT TGTATTCTTT ATCAGGAAAT 1200

TCAGGGAGAC GGGAAGTTAC TATCTACTAA AAGCCAAACA ATTTCTTACA GTTTTACTCT 1260

CTCTACTCTA GAGTAGCTTG GCACTGGCCG TCGTTTTACA ACGTCGTGAC TGGGAAAACC 1320

CTGGCGTTAC CCAACTTAAT CGCCTTGCAG CACATCCCCC TTTCGCCAGC TGGCGTAATA 1380

GCGAAGAGGC CCGCACCGAT CGCCCTTCCC AACAGTTGCG CAGCCTGAAT GGCGAATGGC 1440

GCCTGATGCG GTATTTTCTC CTTACGCATC TGTGCGGTAT TTCACACCGC ATATGGTGCA 1500

CTCTCAGTAC AATCTGCTCT GATGCCGCAT AGTTAAGCCA GCCCCGACAC CCGCCAACAC 1560

CCGCTGACGC GCCCTGACGG GCTTGTCTGC TCCCGGCATC CGCTTACAGA CAAGCTGTGA 1620

CCGTCTCCGG GAGCTGCATG TGTCAGAGGT TTTCACCGTC ATCACCGAAA CGCGCGAGAC 1680

GAAAGGGCCT CGTGATACGC CTATTTTTAT AGGTTAATGT CATGATAATA ATGGTTTCTT 1740

AGACGTCAGG TGGCACTTTT CGGGGAAATG TGCGCGGAAC CCCTATTTGT TTATTTTTCT 1800

AAATACATTC AAATATGTAT CCGCTCATGA GACAATAACC CTGATAAATG CTTCAATAAT 1860

ATTGAAAAAG GAAGAGTATG AGTATTCAAC ATTTCCGTGT CGCCCTTATT CCCTTTTTTG 1920

CGGCATTTTG CCTTCCTGTT TTTGCTCACC CAGAAACGCT GGTGAAAGTA AAAGATGCTG 1980

AAGATCAGTT GGGTGCACGA GTGGGTTACA TCGAACTGGA TCTCAACAGC GGTAAGATCC 2040

TTGAGAGTTT TCGCCCCGAA GAACGTTTTC CAATGATGAG CACTTTTAAA GTTCTGCTAT 2100

GTGGCGCGGT ATTATCCCGT ATTGACGCCG GGCAAGAGCA ACTCGGTCGC CGCATACACT 2160

ATTCTCAGAA TGACTTGGTT GAGTACTCAC CAGTCACAGA AAAGCATCTT ACGGATGGCA 2220

TGACAGTAAG AGAATTATGC AGTGCTGCCA TAACCATGAG TGATAACACT GCGGCCAACT 2280

TACTTCTGAC AACGATCGGA GGACCGAAGG AGCTAACCGC TTTTTTGCAC AACATGGGGG 2340

ATCATGTAAC TCGCCTTGAT CGTTGGGAAC CGGAGCTGAA TGAAGCCATA CCAAACGACG 2400

AGCGTGACAC CACGATGCCT GTAGCAATGG CAACAACGTT GCGCAAACTA TTAACTGGCG 2460

AACTACTTAC TCTAGCTTCC CGGCAACAAT TAATAGACTG GATGGAGGCG GATAAAGTTG 2520

CAGGACCACT TCTGCGCTCG GCCCTTCCGG CTGGCTGGTT TATTGCTGAT AAATCTGGAG 2580

CCGGTGAGCG TGGGTCTCGC GGTATCATTG CAGCACTGGG GCCAGATGGT AAGCCCTCCC 2640

GTATCGTAGT TATCTACACG ACGGGGAGTC AGGCAACTAT GGATGAACGA AATAGACAGA 2700

TCGCTGAGAT AGGTGCCTCA CTGATTAAGC ATTGGTAACT GTCAGACCAA GTTTACTCAT 2760

ATATACTTTA GATTGATTTA AAACTTCATT TTTAATTTAA AAGGATCTAG GTGAAGATCC 2820

TTTTTGATAA TCTCATGACC AAAATCCCTT AACGTGAGTT TTCGTTCCAC TGAGCGTCAG 2880

ACCCCGTAGA AAAGATCAAA GGATCTTCTT GAGATCCTTT TTTTCTGCGC GTAATCTGCT 2940

GCTTGCAAAC AAAAAAACCA CCGCTACCAG CGGTGGTTTG TTTGCCGGAT CAAGAGCTAC 3000

CAACTCTTTT TCCGAAGGTA ACTGGCTTCA GCAGAGCGCA GATACCAAAT ACTGTCCTTC 3060

TAGTGTAGCC GTAGTTAGGC CACCACTTCA AGAACTCTGT AGCACCGCCT ACATACCTCG 3120

CTCTGCTAAT CCTGTTACCA GTGGCTGCTG CCAGTGGCGA TAAGTCGTGT CTTACCGGGT 3180

TGGACTCAAG ACGATAGTTA CCGGATAAGG CGCAGCGGTC GGGCTGAACG GGGGGTTCGT 3240

GCACACAGCC CAGCTTGGAG CGAACGACCT ACACCGAACT GAGATACCTA CAGCGTGAGC 3300

ATTGAGAAAG CGCCACGCTT CCCGAAGGGA GAAAGGCGGA CAGGTATCCG GTAAGCGGCA 3360

GGGTCGGAAC AGGAGAGCGC ACGAGGGAGC TTCCAGGGGG AAACGCCTGG TATCTTTATA 3420

GTCCTGTCGG GTTTCGCCAC CTCTGACTTG AGCGTCGATT TTTGTGATGC TCGTCAGGGG 3480

GGCGGAGCCT ATGGAAAAAC GCCAGCAACG CGGCCTTTTT ACGGTCCTGG CCTTTTGCTG 3540

GCCTTTTGCT CACATGTCTT TCCTGCGTTA TCCCCTGATT CTGTGGATAA CCGTATTACC 3600

GCCTTTGAGT GAGCTGATAC CGCTCGCCGC AGCCGAACCG ACCGAGCGCA GCGAGTCAGT 3660

GAGCGAGGAA GCGGAAGAGC GCCCAATACG CAAACCGCCT CTCCCCGCGC GTTGGCCGAT 3720

TCATTAATGC AGCTGGCACG ACAGGTTTCC CGACTGGAAA GCGGGCAGTG AGCGCAACGC 3780

AATTAATGTG AGTTAGCTCA CTCATTAGGC ACCCCAGGCT TTACACTTTA TGCTTCCGGC 3840

TCGTATGTTG TGTGGAATTG TGAGCGGATA ACAATTTCAC ACAGGAAACA GCTATGACAT 3900

GATTACCG 3908

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GATCCTTATT AGATCTCACC ATCACCATCA CCATTGTTGA G 41

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

TCGACTCAAC AATGGTGATG GTGATGGTGA GATCTAATAA G 41

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3878 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TTGCAGGACA GGATGTGGTG CCCGATGTGA CTAGCTCTTT GCTGCAGGCC GTCCTATCCT 60

CTGGTTCCGA TAAGAGACCC AGAACTCCGG CCCCCCACCG CCCACCGCCA CCCCCATACA 120

TATGTGGTAC GCAAGTAAGA GTGCCTGCGC ATGCCCCATG TGCCCCACCA AGAGcTTTGC 180

ATCCCATACA AGTCCCCAAA GTGGAGAACC GAACCAATTC TTCGCGGGCA GAACAAAAGC 240

TTCTGCACAC GTCTCCACTC GAATTTGGAG CCGGCCGGCG TGTGCAAAAG AGGTGAATCG 300

AACGAAAGAC CCGTGTGTAA AGCCGCGTTT CCAAAATGTA TAAAACCGAG AGCATCTGGC 360
CAATGTGCAT CAGTTGTGGT CAGCAGCAAA ATCAAGTGAA TCATCTCAGT GCAACTAAAG 420
GGGAATTCGA GCTCGGTACC CGGGGATCCT TATTAGATCT CACCATCACC ATCACCATTG 480
TTGAGTCGAC CTGCAGGCAT GCAAGCTATT CGATGCACAC TCACATTCTT CTCCTAATAC 540
GATAATAAAA CTTTCCATGA AAAATATGGA AAAATATATG AAAATTGAGA AATCCAAAAA 600
ACTGATAAAC GCTCTACTTA ATTAAAATAG ATAAATGGGA GCGGCAGGAA TGGCGGAGCA 660
TGGCCAAGTT CCTCCGCCAA TCAGTCGTAA AACAGAAGTC GTGGAAAGCG GATAGAAAGA 720
ATGTTCGATT TGACGGGCAA GCATGTCTGC TATGTGGCGG ATTGCGGAGG AATTGCACTG 780
GAGACCAGCA AGGTTCTCAT GACCAAGAAT ATAGCGGTGA GTGAGCGGGA AGCTCGGTTT 840
CTGTCCAGAT CGAACTCAAA ACTAGTCCAG CCAGTCGCTG TCGAAACTAA TTAAGTtAAT 900
GAGTTTTTCA TGTTAGTTTC GCGCTGAGCA ACAATTAAGT TTATGTTTCA GTTCGGCTTA 960
GATTTCGCTG AAGGACTTGC CACTTTCAAT CAATACTTTA GAACAAAATC AAAACTCATT 1020
CTAATAGCTT GGTGTTCATC TTTTTTTTTA ATGATAAGCA TTTTGTCGTT TATACTTTTT 1080
ATATTTCGAT ATTAAACCAC CTATGAAGTc tATTTTAATC GCCAGATAAG CAATATATTG 1140
TGTAAATATT TGTATTCTTT ATCAGGAAAT TCAGGGAGAC G3GAAGTTAC TATCTACTAA 1200
AAGCCAAACA ATTTCTTACA GTTTTACTCT CTCTACTCTA GAGTAGCTTG GCACTGGCCG 1260
TCGTTTTACA ACGTCGTGAC TGGGAAAACC CTGGCGTTAC CCAACTTAAT CGCCTTGCAG 1320
CACATCCCCC TTTCGCCAGC TGGCGTAATA GCGAAGAGGC CCGCACCGAT CGCCCTTCCC 1380
AACAGTTGCG CAGCCTGAAT GGCGAATGGC GCCTGATGCG GTATTTTCTC CTTACGCATC 1440
TGTGCGGTAT TTCACACCGC ATATGGTGCA CTCTCAGTAC AATCTGCTCT GATGCCGCAT 1500
AGTTAAGCCA GCCCCGACAC CCGCCAACAC CCGCTGACGC GCCCTGACGG GCTTGTCTGC 1560
TCCCGGCATC CGCTTACAGA CAAGCTGTGA CCGTCTCCGG GAGCTGCATG TGTCAGAGGT 1620
TTTCACCGTC ATCACCGAAA CGCGCGAGAC GAAAGGGCCT CGTGATACGC CTATTTTTAT 1680
AGGTTAATGT CATGATAATA ATGGTTTCTT AGACGTCAGG TGGCACTTTT CGGGGAAATG 1740
TGCGCGGAAC CCCTATTTGT TTATTTTTCT AAATACATTC AAATATGTAT CCGCTCATGA 1800
GACAATAACC CTGATAAATG CTTCAATAAT ATTGAAAAAG GAAGAGTATG AGTATTCAAC 1860
ATTTCCGTGT CGCCCTTATT CCCTTTTTTG CGGCATTTTG CCTTCCTGTT TTTGCTCACC 1920
CAGAAACGCT GGTGAAAGTA AAAGATGCTG AAGATCAGTT GGGTGCACGA GTGGGTTACA 1980
TCGAACTGGA TCTCAACAGC GGTAAGATCC TTGAGAGTTT TCGCCCCGAA GAACGTTTTC 2040
CAATGATGAG CACTTTTAAA GTTCTGCTAT GTGGCGCGGT ATTATCCCGT ATTGACGCCG 2100
GGCAAGAGCA ACTCGGTCGC CGCATACACT ATTCTCAGAA TGACTTGGTT GAGTACTCAC 2160
CAGTCACAGA AAAGCATCTT ACGGATGGCA TGACAGTAAG AGAATTATGC AGTGCTGCCA 2220

TAACCATGAG TGATAACACT GCGGCCAACT TACTTCTGAC AACGATCGGA GGACCGAAGG 2280

AGCTAACCGC TTTTTTGCAC AACATGGGGG ATCATGTAAC TCGCCTTGAT CGTTGGGAAC 2340

CGGAGCTGAA TGAAGCCATA CCAAACGACG AGCGTGACAC CACGATGCCT GTAGCAATGG 2400

CAACAACGTT GCGCAAACTA TTAACTGGCG AACTACTTAC TCTAGCTTCC CGGCAACAAT 2460

TAATAGACTG GATGGAGGCG GATAAAGTTG CAGGACCACT TCTGCGCTCG GCCCTTCCGG 2520

CTGGCTGGTT TATTGCTGAT AAATCTGGAG CCGGTGAGCG TGGGTCTCGC GGTATCATTG 2580

CAGCACTGGG GCCAGATGGT AAGCCCTCCC GTATCGTAGT TATCTACACG ACGGGGAGTC 2640

AGGCAACTAT GGATGAACGA AATAGACAGA TCGCTGAGAT AGGTGCCTCA CTGATTAAGC 2700

ATTGGTAACT GTCAGACCAA GTTTACTCAT ATATACTTTA GATTGATTTA AAACTTCATT 2760

TTTAATTTAA AAGGATCTAG GTGAAGATCC TTTTTGATAA TCTCATGACC AAAATCCCTT 2820

AACGTGAGTT TTCGTTCCAC TGAGCGTCAG ACCCCGTAGA AAAGATCAAA GGATCTTCTT 2880

GAGATCCTTT TTTTCTGCGC GTAATCTGCT GCTTGCAAAC AAAAAAACCA CCGCTACCAG 2940

CGGTGGTTTG TTTGCCGGAT CAAGAGCTAC CAACTCTTTT TCCGAAGGTA ACTGGCTTCA 3000

GCAGAGCGCA GATACCAAAT ACTGTCCTTC TAGTGTAGCC GTAGTTAGGC CACCACTTCA 3060

AGAACTCTGT AGCACCGCCT ACATACCTCG CTCTGCTAAT CCTGTTACCA GTGGCTGCTG 3120

CCAGTGGCGA TAAGTCGTGT CTTACCGGGT TGGACTCAAG ACGATAGTTA CCGGATAAGG 3180

CGCAGCGGTC GGGCTGAACG GGGGGTTCGT GCACACAGCC CAGCTTGGAG CGAACGACCT 3240

ACACCGAACT GAGATACCTA CAGCGTGAGC ATTGAGAAAG CGCCACGCTT CCCGAAGGGA 3300

GAAAGGCGGA CAGGTATCCG GTAAGCGGCA GGGTCGGAAC AGGAGAGCGC ACGAGGGAGC 3360

TTCCAGGGGG AAACGCCTGG TATCTTTATA GTCCTGTCGG GTTTCGCCAC CTCTGACTTG 3420

AGCGTCGATT TTTGTGATGC TCGTCAGGGG GGCGGAGCCT ATGGAAAAAC GCCAGCAACG 3480

CGGCCTTTTT ACGGTCCTGG CCTTTTGCTG GCCTTTTGCT CACATGTCTT TCCTGCGTTA 3540

TCCCCTGATT CTGTGGATAA CCGTATTACC GCCTTTGAGT GAGCTGATAC CGCTCGCCGC 3600

AGCCGAACCG ACCGAGCGCA GCGAGTCAGT GAGCGAGGAA GCGGAAGAGC GCCCAATACG 3660

CAAACCGCCT CTCCCCGCGC GTTGGCCGAT TCATTAATGC AGCTGGCACG ACAGGTTTCC 3720

CGACTGGAAA GCGGGCAGTG AGCGCAACGC AATTAATGTG AGTTAGCTCA CTCATTAGGC 3780

ACCCCAGGCT TTACACTTTA TGCTTCCGGC TCGTATGTTG TGTGGAATTG TGAGCGGATA 3840

ACAATTTCAC ACAGGAAACA GCTATGACAT GATTACCG 3878

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GATCCTTATT AGATCTGCTT GGCGCCATCC TCAATTTGGG GGTTGAG 47

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

TCGACTCAAC CCCCAAATTG AGGATGGCGC CAAGCAGATC TAATAAG 47

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3883 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

TTGCAGGACA GGATGTGGTG CCCGATGTGA CTAGCTCTTT GCTGCAGGCC GTCCTATCCT 60

TGGTTCCGAT AAGAGACCCA GAACTCCGGC CCCCCACCGC CCACCGCCAC CCCCATACAT 120

ATGTGGTACG CAAGTAAGAG TGCCTGCGCA TGCCCCATGT GCCCCACCAA GAGCTTTGCA 180

TCCCATACAA GTCCCCAAAG TGGAGAACCG AACCAATTCT TCGCGGGCAG AACAAAAGCT 240

TCTGCACACG TCTCCACTCG AATTTGGAGC CGGCCGGCGT GTGCAAAAGA GGTGAATCGA 300

ACGAAAGACC CGTGTGTAAA GCCGCGTTTC CAAAATGTAT AAAACCGAGA GCATCTGGCC 360

AATGTGCATC AGTTGTGGTC AGCAGCAAAA TCAAGTGAAT CATCTCAGTG CAACTAAAGG 420
GGAATTCGAG CTCGGTACCC GGGGATCCTT ATTAGATCTG CTTGGCGCCA TCCTCAATTT 480
GGGGGTTGAG TCGACCTGCA GGCATGCAAG CTATTCGATG CACACTCACA TTCTTCTCCT 540
AATACGATAA TAAAACTTTC CATGAAAAAT ATGGAAAAAT ATATGAAAAT TGAGAAATCC 600
AAAAAACTGA TAAACGCTCT ACTTAATTAA AATAGATAAA TGGGAGCGGC AGGAATGGCG 660
GAGCATGGCC AAGTTCCTCC GCCAATCAGT CGTAAAACAG AAGTCGTGGA AAGCGGATAG 720
AAAGAATGTT CGATTTGACG GGCAAGCATG TCTGCTATGT GGCGGATTGC GGAGGAATTG 780
CACTGGAGAC CAGCAAGGTT CTCATGACCA AGAATATAGC GGTGAGTGAG CGGGAAGCTC 840
GGTTTCTGTC CAGATCGAAC TCAAAACTAG TCCAGCCAGT CGCTGTCGAA ACTAATTAAG 900
TTAATGAGTT TTTCATGTTA GTTTCGCGCT GAGCAACAAT TAAGTTTATG TTTCAGTTCG 960
GCTTAGATTT CGCTGAAGGA CTTGCCACTT TCAATCAATA CTTTAGAACA AAATCAAAAC 1020
TCATTCTAAT AGCTTGGTGT TCATCTTTTT TTTTAATGAT AAGCATTTTG TCGTTTATAC 1080
TTTTTATATT TCGATATTAA ACCACCTATG AAGTCtATTT TAATCGCCAG ATAAGCAATA 1140
TATTGTGTAA ATATTTGTAT TCTTTATCAG GAAATTCAGG GAGACGGGAA GTTACTATCT 1200
ACTAAAAGCC AAACAATTTC TTACAGTTTT ACTCTCTCTA CTCTAGAGTA GCTTGGCACT 1260
GGCCGTCGTT TTACAACGTC GTGACTGGGA AAACCCTGGC GTTACCCAAC TTAATCGCCT 1320
TGCAGCACAT CCCCCTTTCG CCAGCTGGCG TAATAGCGAA GAGGCCCGCA CCGATCGCCC 1380
TTCCCAACAG TTGCGCAGCC TGAATGGCGA ATGGCGCCTG ATGCGGTATT TTCTCCTTAC 1440
GCATCTGTGC GGTATTTCAC ACCGCATATG GTGCACTCTC AGTACAATCT GCTCTGATGC 1500
CGCATAGTTA AGCCAGCCCC GACACCCGCC AACACCCGCT GACGCGCCCT GACGGGCTTG 1560
TCTGCTCCCG GCATCCGCTT ACAGACAAGC TGTGACCGTC TCCGGGAGCT GCATGTGTCA 1620
GAGGTTTTCA CCGTCATCAC CGAAACGCGC GAGACGAAAG GGCCTCGTGA TACGCCTATT 1680
TTTATAGGTT AATGTCATGA TAATAATGGT TTCTTAGACG TCAGGTGGCA CTTTTCGGGG 1740
AAATGTGCGC GGAACCCCTA TTTGTTTATT TTTCTAAATA CATTCAAATA TGTATCCGCT 1800
CATGAGACAA TAACCCTGAT AAATGCTTCA ATAATATTGA AAAAGGAAGA GTATGAGTAT 1860
TCAACATTTC CGTGTCGCCC TTATTCCCTT TTTTGCGGCA TTTTGCCTTC CTGTTTTTGC 1920
TCACCCAGAA ACGCTGGTGA AAGTAAAAGA TGCTGAAGAT CAGTTGGGTG CACGAGTGGG 1980
TTACATCGAA CTGGATCTCA ACAGCGGTAA GATCCTTGAG AGTTTTCGCC CCGAAGAACG 2040
TTTTCCAATG ATGAGCACTT TTAAAGTTCT GCTATGTGGC GCGGTATTAT CCCGTATTGA 2100
CGCCGGGCAA GAGCAACTCG GTCGCCGCAT ACACTATTCT CAGAATGACT TGGTTGAGTA 2160
CTCACCAGTC ACAGAAAAGC ATCTTACGGA TGGCATGACA GTAAGAGAAT TATGCAGTGC 2220
TGCCATAACC ATGAGTGATA ACACTGCGGC CAACTTACTT CTGACAACGA TCGGAGGACC 2280

GAAGGAGCTA ACCGCTTTTT TGCACAACAT GGGGGATCAT GTAACTCGCC TTGATCGTTG 2340

GGAACCGGAG CTGAATGAAG CCATACCAAA CGACGAGCGT GACACCACGA TGCCTGTAGC 2400

AATGGCAACA ACGTTGCGCA AACTATTAAC TGGCGAACTA CTTACTCTAG CTTCCCGGCA 2460

ACAATTAATA GACTGGATGG AGGCGGATAA AGTTGCAGGA CCACTTCTGC GCTCGGCCCT 2520

TCCGGCTGGC TGGTTTATTG CTGATAAATC TGGAGCCGGT GAGCGTGGGT CTCGCGGTAT 2580

CATTGCAGCA CTGGGGCCAG ATGGTAAGCC CTCCCGTATC GTAGTTATCT ACACGACGGG 2640

GAGTCAGGCA ACTATGGATG AACGAAATAG ACAGATCGCT GAGATAGGTG CCTCACTGAT 2700

TAAGCATTGG TAACTGTCAG ACCAAGTTTA CTCATATATA CTTTAGATTG ATTTAAAACT 2760

TCATTTTTAA TTTAAAAGGA TCTAGGTGAA GATCCTTTTT GATAATCTCA TGACCAAAAT 2820

CCCTTAACGT GAGTTTTCGT TCCACTGAGC GTCAGACCCC GTAGAAAAGA TCAAAGGATC 2880

TTCTTGAGAT CCTTTTTTTC TGCGCGTAAT CTGCTGCTTG CAAACAAAAA AACCACCGCT 2940

ACCAGCGGTG GTTTGTTTGC CGGATCAAGA GCTACCAACT CTTTTTCCGA AGGTAACTGG 3000

CTTCAGCAGA GCGCAGATAC CAAATACTGT CCTTCTAGTG TAGCCGTAGT TAGGCCACCA 3060

CTTCAAGAAC TCTGTAGCAC CGCCTACATA CCTCGCTCTG CTAATCCTGT TACCAGTGGC 3120

TGCTGCCAGT GGCGATAAGT CGTGTCTTAC CGGGTTGGAC TCAAGACGAT AGTTACCGGA 3180

TAAGGCGCAG CGGTCGGGCT GAACGGGGGG TTCGTGCACA CAGCCCAGCT TGGAGCGAAC 3240

GACCTACACC GAACTGAGAT ACCTACAGCG TGAGCATTGA GAAAGCGCCA CGCTTCCCGA 3300

AGGGAGAAAG GCGGACAGGT ATCCGGTAAG CGGCAGGGTC GGAACAGGAG AGCGCACGAG 3360

GGAGCTTCCA GGGGGAAACG CCTGGTATCT TTATAGTCCT GTCGGGTTTC GCCACCTCTG 3420

ACTTGAGCGT CGATTTTTGT GATGCTCGTC AGGGGGGCGG AGCCTATGGA AAAACGCCAG 3480

CAACGCGGCC TTTTTACGGT CCTGGCCTTT TGCTGGCCTT TTGCTCACAT GTCTTTCCTG 3540

CGTTATCCCC TGATTCTGTG GATAACCGTA TTACCGCCTT TGAGTGAGCT GATACCGCTC 3600

GCCGCAGCCG AACCGACCGA GCGCAGCGAG TCAGTGAGCG AGGAAGCGGA AGAGCGCCCA 3660

ATACGCAAAC CGCCTCTCCC CGCGCGTTGG CCGATTCATT AATGCAGCTG GCACGACAGG 3720

TTTCCCGACT GGAAAGCGGG CAGTGAGCGC AACGCAATTA ATGTGAGTTA GCTCACTCAT 3780

TAGGCACCCC AGGCTTTACA CTTTATGCTT CCGGCTCGTA TGTTGTGTGG AATTGTGAGC 3840

GGATAACAAT TTCACACAGG AAACAGCTAT GACATGATTA CCG 3883

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 879 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GAATTCATGG GCCACACACG GAGGCAGGGA ACATCACCAT CCAAGTGTCC ATACCTCAAT 60

TTCTTTCAGC TCTTGGTGCT GGCTGGTCTT TCTCACTTCT GTTCAGGTGT TATCCACGTG 120

ACCAAGGAAG TGAAAGAAGT GGCAACGCTG TCCTGTGGTC ACAATGTTTC TGTTGAAGAG 180

CTGGCACAAA CTCGCATCTA CTGGCAAAAG GAGAAGAAAA TGGTGCTGAC TATGATGTCT 240

GGGGACATGA ATATATGGCC CGAGTACAAG AACCGGACCA TCTTTGATAT CACTAATAAC 300

CTCTCCATTG TGATCCTGGC TCTGCGCCCA TCTGACGAGG GCACATACGA GTGTGTTGTT 360

CTGAAGTATG AAAAAGACGC TTTCAAGCGG GAACACCTGG CTGAAGTGAC GTTATCAGTC 420

AAAGCTGACT TCCCTACACC TAGTATATCT GACTTTGAAA TTCCAACTTC TAATATTAGA 480

AGGATAATTT GCTCAACCTC TGGAGGTTTT CCAGAGCCTC ACCTCTCCTG GTTGGAAAAT 540

GGAGAAGAAT TAAATGCCAT CAACACAACA GTTTCCCAAG ATCCTGAAAC TGAGCTCTAT 600

GCTGTTAGCA GCAAACTGGA TTTCAATATG ACAACCAACC ACAGCTTCAT GTGTCTCATC 660

AAGTATGGAC ATTTAAGAGT GAATCAGACC TTCAACTGGA ATACAACCAA GCAAGAGCAT 720

TTTCCTGATA ACCTGCTCCC ATCCTGGGCC ATTACCTTAA TCTCAGTAAA TGGAATTTTT 780

GTGATATGCT GCCTGACCTA CTGCTTTGCC CCAAGATGCA GAGAGAGAAG GAGGAATGAG 840

AGATTGAGAA GGGAAAGTGT ACGCCCTGTA TAAGGATTC 879

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 738 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

GAATTCATGG GCCACACACG GAGGCAGGGA ACATCACCAT CCAAGTGTCC ATACCTCAAT 60

TTCTTTCAGC TCTTGGTGCT GGCTGGTCTT TCTCACTTCT GTTCAGGTGT TATCCACGTG 120

ACCAAGGAAG TGAAAGAAGT GGCAACGCTG TCCTGTGGTC ACAATGTTTC TGTTGAAGAG 180

CTGGCACAAA CTCGCATCTA CTGGCAAAAG GAGAAGAAAA TGGTGCTGAC TATGATGTCT 240

GGGGACATGA ATATATGGCC CGAGTACAAG AACCGGACCA TCTTTGATAT CACTAATAAC 300

CTCTCCATTG TGATCCTGGC TCTGCGCCCA TCTGACGAGG GCACATACGA GTGTGTTGTT 360

CTGAAGTATG AAAAAGACGC TTTCAAGCGG GAACACCTGG CTGAAGTGAC GTTATCAGTC 420

AAAGCTGACT TCCCTACACC TAGTATATCT GACTTTGAAA TTCCAACTTC TAATATTAGA 480

AGGATAATTT GCTCAACCTC TGGAGGTTTT CCAGAGCCTC ACCTCTCCTG GTTGGAAAAT 540

GGAGAAGAAT TAAATGCCAT CAACACAACA GTTTCCCAAG ATCCTGAAAC TGAGCTCTAT 600

GCTGTTAGCA GCAAACTGGA TTTCAATATG ACAACCAACC ACAGCTTCAT GTGTCTCATC 660

AAGTATGGAC ATTTAAGAGT GAATCAGACC TTCAACTGGA ATACAACCAA GCAAGAGCAT 720

TTTCCTGATA ACGGATTC 738

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1002 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

GAGCTCATGG ATCCCCAGTG CACTATGGGA CTGAGTAACA TTCTCTTTGT GATGGCCTTC 60

CTGCTCTCTG GTGCTGCTCC TCTGAAGATT CAAGCTTATT TCAATGAGAC TGCAGACCTG 120

CCATGCCAAT TTGCAAACTC TCAAAACCAA AGCCTGAGTG AGCTAGTAGT ATTTTGGCAG 180

GACCAGGAAA ACTTGGTTCT GAATGAGGTA TACTTAGGCA AAGAGAAATT TGACAGTGTT 240

CATTCCAAGT ATATGGGCCG CACAAGTTTT GATTCGGACA GTTGGACCCT GAGACTTCAC 300

AATCTTCAGA TCAAGGACAA GGGCTTGTAT CAATGTATCA TCCATCACAA AAAGCCCACA 360

GGAATGATTC GCATCCACCA GATGAATTCT GAACTGTCAG TGCTTGCTAA CTTCAGTCAA 420

CCTGAAATAG TACCAATTTC TAATATAACA GAAAATGTGT ACATAAATTT GACCTGCTCA 480

TCTATACACG GTTACCCAGA ACCTAAGAAG ATGAGTGTTT TGCTAAGAAC CAAGAATTCA 540

ACTATCGAGT ATGATGGTAT TATGCAGAAA TCTCAAGATA ATGTCACAGA ACTGTACGAC 600

GTTTCCATCA GCTTGTCTGT TTCATTCCCT GATGTTACGA GCAATATGAC CATCTTCTGT 660

ATTCTGGAAA CTGACAAGAC GCGGCTTTTA TCTTCACCTT TCTCTATAGA GCTTGAGGAC 720

CCTCAGCCTC CCCCAGACCA CATTCCTTGG ATTACAGCTG TACTTCCAAC AGTTATTATA 780

TGTGTGATGG TTTTCTGTCT AATTCTATGG AAATGGAAGA AGAAGAAGCG GCCTCGCAAC 840

TCTTATAAAT GTGGAACCAA CACAATGGAG AGGGAAGAGA GTGAACAGAC CAAGAAAAGA 900

GAAAAAATCC ATATACCTGA AAGATCTGAT GAAGCCCAGC GTGTTTTTAA AAGTTCGAAG 960

ACATCTTCAT GCGACAAAAG TGATACATGT TTTTAAGGGC CC 1002

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 751 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GAGCTCATGG ATCCCCAGTG CACTATGGGA CTGAGTAACA TTCTCTTTGT GATGGCCTTC 60

CTGCTCTCTG GTGCTGCTCC TCTGAAGATT CAAGCTTATT TCAATGAGAC TGCAGACCTG 120

CCATGCCAAT TTGCAAACTC TCAAAACCAA AGCCTGAGTG AGCTAGTAGT ATTTTGGCAG 180

GACCAGGAAA ACTTGGTTCT GAATGAGGTA TACTTAGGCA AAGAGAAATT TGACAGTGTT 240

CATTCCAAGT ATATGGGCCG CACAAGTTTT GATTCGGACA GTTGGACCCT GAGACTTCAC 300

AATCTTCAGA TCAAGGACAA GGGCTTGTAT CAATGTATCA TCCATCACAA AAAGCCCACA 360

GGAATGATTC GCATCCACCA GATGAATTCT GAACTGTCAG TGCTTGCTAA CTTCAGTCAA 420

CCTGAAATAG TACCAATTTC TAATATAACA GAAAATGTGT ACATAAATTT GACCTGCTCA 480

TCTATACACG GTTACCCAGA ACCTAAGAAG ATGAGTGTTT TGCTAAGAAC CAAGAATTCA 540

ACTATCGAGT ATGATGGTAT TATGCAGAAA TCTCAAGATA ATGTCACAGA ACTGTACGAC 600

GTTTCCATCA GCTTGTCTGT TTCATTCCCT GATGTTACGA GCAATATGAC CATCTTCTGT 660

ATTCTGGAAA CTGACAAGAC GCGGCTTTTA TCTTCACCTT TCTCTATAGA GCTTGAGGAC 720

CCTCAGCCTC CCCCAGACCA CATTGGGGCC C 751

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1611 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

GAATTCATGG CTCCCAGCAG CCCCCGGCCC GCGCTGCCCG CACTCCTGGT CCTGCTCGGG 60

GCTCTGTTCC CAGGACCTGG CAATGCCCAG ACATCTGTGT CCCCCTCAAA AGTCATCCTG 120

CCCCGGGGAG GCTCCGTGCT GGTGACATGC AGCACCTCCT GTGACCAGCC CAAGTTGTTG 180

GGCATAGAGA CCCCGTTGCC TAAAAAGGAG TTGCTCCTGC CTGGGAACAA CCGGAAGGTG 240

TATGAACTGA GCAATGTGCA AGAAGATAGC CAACCAATGT GCTATTCAAA CTGCCCTGAT 300

GGGCAGTCAA CAGCTAAAAC CTTCCTCACC GTGTACTGGA CTCCAGAACG GGTGGAACTG 360

GCACCCCTCC CCTCTTGGCA GCCAGTGGGC AAGAACCTTA CCCTACGCTG CCAGGTGGAG 420

GGTGGGGCAC CCCGGGCCAA CCTCACCGTG GTGCTGCTCC GTGGGGAGAA GGAGCTGAAA 480

CGGGAGCCAG CTGTGGGGGA GCCCGCTGAG GTCACGACCA CGGTGCTGGT GAGGAGAGAT 540

CACCATGGAG CCAATTTCTC GTGCCGCACT GAACTGGACC TGCGGCCCCA AGGGCTGGAG 600

CTGTTTGAGA ACACCTCGGC CCCCTACCAG CTCCAGACCT TTGTCCTGCC AGCGACTCCC 660

CCACAACTTG TCAGCCCCCG GGTCCTAGAG GTGGACACGC AGGGGACCGT GGTCTGTTCC 720

CTGGACGGGC TGTTCCCAGT CTCGGAGGCC CAGGTCCACC TGGCACTGGG GGACCAGAGG 780

TTGAACCCCA CAGTCACCTA TGGCAACGAC TCCTTCTCGG CCAAGGCCTC AGTCAGTGTG 840

ACCGCAGAGG ACGAGGGCAC CCAGCGGCTG ACGTGTGCAG TAATACTGGG GAACCAGAGC 900

CAGGAGACAC TGCAGACAGT GACCATCTAC AGCTTTCCGG CGCCCAACGT GATTCTGACG 960

AAGCCAGAGG TCTCAGAAGG GACCGAGGTG ACAGTGAAGT GTGAGGCCCA CCCTAGAGCC 1020

AAGGTGACGC TGAATGGGGT TCCAGCCCAG CCACTGGGCC CGAGGGCCCA GCTCCTGCTG 1080

AAGGCCACCC CAGAGGACAA CGGGCGCAGC TTCTCCTGCT CTGCAACCCT GGAGGTGGCC 1140

GGCCAGCTTA TACACAAGAA CCAGACCCGG GAGCTTCGTG TCCTGTATGG CCCCCGACTG 1200

GACGAGAGGG ATTGTCCGGG AAACTGGACG TGGCCAGAAA ATTCCCAGCA GACTCCAATG 1260

TGCCAGGCTT GGGGGAACCC ATTGCCCGAG CTCAAGTGTC TAAAGGATGG CACTTTCCCA 1320

CTGCCCATCG GGGAATCAGT GACTGTCACT CGAGATCTTG AGGGCACCTA CCTCTGTCGG 1380

GCCAGGAGCA CTCAAGGGGA GGTCACCCGC GAGGTGACCG TGAATGTGCT CTCCCCCCGG 1440

TATGAGATTG TCATCATCAC TGTGGTAGCA GCCGCAGTCA TAATGGGCAC TGCAGGCCTC 1500

AGCACGTACC TCTATAACCG CCAGCGGAAG ATCAAGAAAT ACAGACTACA ACAGGCCCAA 1560

AAAGGGACCC CCATGAAACC GAACACACAA GCCACGCCTC CCTGAGGATC C 1611

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1452 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

GAATTCATGG CTCCCAGCAG CCCCCGGCCC GCGCTGCCCG CACTCCTGGT CCTGCTCGGG 60

GCTCTGTTCC CAGGACCTGG CAATGCCCAG ACATCTGTGT CCCCCTCAAA AGTCATCCTG 120

CCCCGGGGAG GCTCCGTGCT GGTGACATGC AGCACCTCCT GTGACCAGCC CAAGTTGTTG 180

GGCATAGAGA CCCCGTTGCC TAAAAAGGAG TTGCTCCTGC CTGGGAACAA CCGGAAGGTG 240

TATGAACTGA GCAATGTGCA AGAAGATAGC CAACCAATGT GCTATTCAAA CTGCCCTGAT 300

GGGCAGTCAA CAGCTAAAAC CTTCCTCACC GTGTACTGGA CTCCAGAACG GGTGGAACTG 360

GCACCCCTCC CCTCTTGGCA GCCAGTGGGC AAGAACCTTA CCCTACGCTG CCAGGTGGAG 420

GGTGGGGCAC CCCGGGCCAA CCTCACCGTG GTGCTGCTCC GTGGGGAGAA GGAGCTGAAA 480

CGGGAGCCAG CTGTGGGGGA GCCCGCTGAG GTCACGACCA CGGTGCTGGT GAGGAGAGAT 540

CACCATGGAG CCAATTTCTC GTGCCGCACT GAACTGGACC TGCGGCCCCA AGGGCTGGAG 600

CTGTTTGAGA ACACCTCGGC CCCCTACCAG CTCCAGACCT TTGTCCTGCC AGCGACTCCC 660

CCACAACTTG TCAGCCCCCG GGTCCTAGAG GTGGACACGC AGGGGACCGT GGTCTGTTCC 720

CTGGACGGGC TGTTCCCAGT CTCGGAGGCC CAGGTCCACC TGGCACTGGG GGACCAGAGG 780

TTGAACCCCA CAGTCACCTA TGGCAACGAC TCCTTCTCGG CCAAGGCCTC AGTCAGTGTG 840

ACCGCAGAGG ACGAGGGCAC CCAGCGGCTG ACGTGTGCAG TAATACTGGG GAACCAGAGC 900

CAGGAGACAC TGCAGACAGT GACCATCTAC AGCTTTCCGG CGCCCAACGT GATTCTGACG 960

AAGCCAGAGG TCTCAGAAGG GACCGAGGTG ACAGTGAAGT GTGAGGCCCA CCCTAGAGCC 1020

AAGGTGACGC TGAATGGGGT TCCAGCCCAG CCACTGGGCC CGAGGGCCCA GCTCCTGCTG 1080

AAGGCCACCC CAGAGGACAA CGGGCGCAGC TTCTCCTGCT CTGCAACCCT GGAGGTGGCC 1140

GGCCAGCTTA TACACAAGAA CCAGACCCGG GAGCTTCGTG TCCTGTATGG CCCCCGACTG 1200

GACGAGAGGG ATTGTCCGGG AAACTGGACG TGGCCAGAAA ATTCCCAGCA GACTCCAATG 1260

TGCCAGGCTT GGGGGAACCC ATTGCCCGAG CTCAAGTGTC TAAAGGATGG CACTTTCCCA 1320

CTGCCCATCG GGGAATCAGT GACTGTCACT CGAGATCTTG AGGGCACCTA CCTCTGTCGG 1380

GCCAGGAGCA CTCAAGGGGA GGTCACCCGC GAGGTGACCG TGAATGTGCT CTCCCCCCGG 1440

TATGAGGGAT CC 1452

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 726 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GAGCTCATGG TTGCTGGGAG CGACGCGGGG CGGGCCCTGG GGGTCCTCAG CGTGGTCTGC 60

CTGCTGCACT GCTTTGGTTT CATCAGCTGT TTTTCCCAAC AAATATATGG TGTTGTGTAT 120

GGGAATGTAA CTTTCCATGT ACCAAGCAAT GTGCCTTTAA AAGAGGTCCT ATGGAAAAAA 180

CAAAAGGATA AAGTTGCAGA ACTGGAAAAT TCTGAATTCA GAGCTTTCTC ATCTTTTAAA 240

AATAGGGTTT ATTTAGACAC TGTGTCAGGT AGCCTCACTA TCTACAACTT AACATCATCA 300

GATGAAGATG AGTATGAAAT GGAATCGCCA AATATTACTG ATACCATGAA GTTCTTTCTT 360

TATGTGCTTG AGTCTCTTCC ATCTCCCACA CTAACTTGTG CATTGACTAA TGGAAGCATT 420

GAAGTCCAAT GCATGATACC AGAGCATTAC AACAGCCATC GAGGACTTAT AATGTACTCA 480

TGGGATTGTC CTATGGAGCA ATGTAAACGT AACTCAACCA GTATATATTT TAAGATGGAA 540

AATGATCTTC CACAAAAAAT ACAGTGTACT CTTAGCAATC CATTATTTAA TACAACATCA 600

TCAATCATTT TGACAACCTG TATCCCAAGC AGCGGTCATT CAAGACACAG ATATGCACTT 660

ATACCCATAC CATTAGCAGT AATTACAACA TGTATTGTGC TGTATATGAA TGTTCTTTAA 720

GGATCC 726

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 657 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

GAGCTCATGG TTGCTGGGAG CGACGCGGGG CGGGCCCTGG GGGTCCTCAG CGTGGTCTGC 60

CTGCTGCACT GCTTTGGTTT CATCAGCTGT TTTTCCCAAC AAATATATGG TGTTGTGTAT 120

GGGAATGTAA CTTTCCATGT ACCAAGCAAT GTGCCTTTAA AAGAGGTCCT ATGGAAAAAA 180

CAAAAGGATA AAGTTGCAGA ACTGGAAAAT TCTGAATTCA GAGCTTTCTC ATCTTTTAAA 240

AATAGGGTTT ATTTAGACAC TGTGTCAGGT AGCCTCACTA TCTACAACTT AACATCATCA 300

GATGAAGATG AGTATGAAAT GGAATCGCCA AATATTACTG ATACCATGAA GTTCTTTCTT 360

TATGTGCTTG AGTCTCTTCC ATCTCCCACA CTAACTTGTG CATTGACTAA TGGAAGCATT 420

GAAGTCCAAT GCATGATACC AGAGCATTAC AACAGCCATC GAGGACTTAT AATGTACTCA 480

TGGGATTGTC CTATGGAGCA ATGTAAACGT AACTCAACCA GTATATATTT TAAGATGGAA 540

AATGATCTTC CACAAAAAAT ACAGTGTACT CTTAGCAATC CATTATTTAA TACAACATCA 600

TCAATCATTT TGACAACCTG TATCCCAAGC AGCGGTCATT CAAGACACAG AGGATCC 657

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

Glu Gln Leu Glu Ser Ile Ile Asn Phe Glu Lys Leu Thr Glu Trp Thr
1 5 10 15

Ser Ser Asn Val Met Glu Glu Arg
20

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

Asp Leu Arg Gly Tyr Val Tyr Gln Gly Leu
1 5 10

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

Phe Arg Ile Gly Cys Arg His Ser Arg
1 5

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

Ile Leu Lys Glu Pro Val His Gly Val
1 5

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

ATATGGATCC TCACCATCTC AGGGTGAGGG GC
32

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

Arg Gly Tyr Val Tyr Gln Gly Leu Lys Ser
1 5 10

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

Phe Ala Pro Gly Asn Tyr Pro Ala Leu
1 5

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

Leu Ser Pro Phe Pro Phe Asp Leu
1 5

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

Gln Leu Ser Pro Phe Pro Phe Asp Leu
1 5

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

Ile Leu Lys Glu Pro Val His Gly Val
1 5

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

Tyr Met Asn Gly Thr Met Ser Gln Val
1 5

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

Gly Ile Leu Gly Phe Val Phe Thr Leu
1 5

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

Phe Leu Pro Ser Asp Phe Phe Pro Ser Val
1 5 10

WE CLAIM:

1. A synthetic antigen presenting cell line for use with T-cell lymphocytes comprising:

a) a Class I MHC heavy chain gene operably linked to a first promoter and capable of expressing a Class I MHC heavy chain;

b) a β-2 microglobulin gene operably linked to a second promoter and capable of expressing a β-2 microglobulin that with the MHC heavy chain forms MHC molecules; and

c) a gene for an assisting molecule operably linked to a third promoter and capable of expressing an assisting molecule that interacts with a molecule on the T-cell lymphocytes;

at least one of the MHC gene, β-2 microglobulin gene and assisting molecule genes not being present in the cells from which the cell line is derived, such that the MHC molecules bind to a peptide, and the MHC molecules and assisting molecules are presented on the surface of the cell in sufficient numbers to activate a population of T-cell lymphocytes against the peptide when the peptide is bound to the MHC molecules.

2. The cell line of claim 1 wherein the assisting molecule is a costimulatory molecule.

3. The cell line of claim 2 wherein the costimulatory molecule is B7.1 or B7.2.

4. The cell line of claim 1 wherein the cell line is derived from a first species and the MHC heavy chain gene is from a second species.

5. The cell line of claim 4 wherein the first species is a poikilotherm and the second species is a homeotherm.

6. The cell line of claim 1 wherein the assisting molecule is an adhesion molecule.

7. The cell line of claim 6 wherein the adhesion molecule is ICAM-1, ICAM-2, ICAM-3 or LFA-3.

8. The cell line of claim 1 having a gene for a first assisting molecule and a gene for a second assisting molecule.

9. The cell line of claim 8 wherein the first assisting molecule is a costimulatory molecule and the second assisting molecule is an adhesion molecule.

10. The cell line of claim 1 wherein at least one promoter is inducible.

11. The cell line of claim 10 wherein the first promoter is inducible.

12. The cell line of claim 1 wherein the peptide is bound to the MHC molecules within the cell.

13. The cell line of claim 1 wherein the MHC molecules are presented empty on the surface of the cell.

14. A stable poikilotherm cell line used to stimulate human T-cell lymphocytes comprising:

a) a Class I MHC gene operably linked to a first inducible promoter and capable of expressing a Class I MHC heavy chain;

b) a β-2 microglobulin gene operably linked to a second promoter and capable of expressing a β-2 microglobulin that forms MHC molecules with the MHC heavy chain; and

c) a gene for an assisting molecule operably linked to a third promoter and capable of expressing an assisting molecule that interacts with a molecule on the T-cell lymphocytes;

such that the cell assembles empty MHC molecules that are capable of binding to a peptide, and the MHC molecules and assisting molecules are presented on the surface of the cells in sufficient numbers to activate a population of T-cell lymphocytes against the peptide when the peptide is bound to the MHC molecules.

15. The cell line of claim 14 wherein the assisting molecule is a costimulatory molecule.

16. The cell line of claim 15 wherein the costimulatory molecule is B7.1 or B7.2.

17. The cell line of claim 14 wherein the assisting molecule is an adhesion molecule.

18. The cell line of claim 17 wherein the adhesion molecule is ICAM-1.

19. The cell line of claim 14 having a gene for a first assisting molecule and a gene for a second assisting molecule.

20. The cell line of claim 19 wherein the first assisting molecule is a costimulatory molecule and the second assisting molecule is an adhesion molecule.

21. A stable poikilotherm cell line used to stimulate human T-cell lymphocytes comprising:

a) a Class I MHC gene operably linked to a first promoter and capable of expressing a Class I MHC heavy chain;

b) a β-2 microglobulin gene operably linked to a second promoter and capable of expressing a β-2 microglobulin that forms MHC molecules with the MHC heavy chain; and

c) a gene for a costimulatory molecule operably linked to a third promoter and capable of expressing a costimulatory molecule that interacts with a molecule on the T-cell lymphocytes;

d) a gene for an adhesion molecule operably linked to a fourth promoter and capable of expressing an adhesion molecule that interacts with a cooperative adhesion molecule on the T-cell lymphocytes;

such that the cell is capable of assembling the MHC heavy chain and the β-2 microglobulin into MHC molecules that bind to a peptide, and transporting MHC molecules, costimulatory molecules and adhesion molecules

to the surface of the cell in sufficient numbers to activate a population of T-cell lymphocytes against the peptide when the peptide is bound to the MHC molecules.

22. The cell line of claim 21 wherein the costimulatory molecule is B7.1 or B7.2.

23. The cell line of claim 21 wherein the adhesion molecule is ICAM-1, ICAM-2, ICAM-3 or LFA-3.

24. The cell line of claim 21 wherein at least one of the promoters is inducible.

25. The cell line of claim 21 wherein the peptide is bound to the MHC molecules within the cell.

26. The cell line of claim 21 wherein the MHC molecules are presented empty on the surface of the cell.

27. Fragments of cells of claim 1 having MHC molecules and assisting molecules operably associated such that they activate the population of T-cell lymphocytes.

28. The cell fragment of claim 27 wherein the MHC molecules are empty.

29. The cell fragment of claim 27 wherein the peptide is bound to the MHC molecules.

30. Fragments of cells of claim 21 having MHC molecules, costimulatory molecules and adhesion molecules operably associated such that they activate the population of T-cell lymphocytes.

31. The cell fragment of claim 27 wherein the peptide is bound to the MHC molecules.

32. A method of producing a synthetic antigen presenting a cell line comprising:

a) establishing of a culture of cells;

b) transfecting the culture with an expressible Class I MHC heavy chain gene operably linked to a first promoter;

c) transfecting the culture with an expressible β-2 microglobulin gene operably linked to a second promoter; and

d) transfecting the culture with an expressible assisting molecule gene operably linked to a third promoter.

33. The method of claim 32 wherein the assisting molecule is a costimulatory molecule.

34. The method of claim 33 wherein the costimulatory molecule is B7.1 or B7.2.

35. The method of claim 32 wherein the cell line is derived from a first species and the MHC heavy chain gene is from a second species.

36. The method of claim 35 wherein the first species is a poikilotherm and the second species is a homeotherm.

37. The method of claim 32 wherein the assisting molecule is an adhesion molecule.

38. The method of claim 37 wherein the adhesion molecule is ICAM-1, ICAM-2, ICAM-3 or LFA-3.

39. The method of claim 32 including the step of transfecting the culture with a gene for a second assisting molecule.

40. The method of claim 39 wherein the first assisting molecule is a costimulatory molecule and the second assisting molecule is an adhesion molecule.

41. The method of claim 32 wherein at least one of the promoters is inducible.

42. A method of producing a synthetic antigen presenting cell line comprising:

a) establishing a culture of cells lacking a gene to at least one of: Class I MHC heavy chain, β-2 microglobulin and assisting molecule; and

b) transfecting the culture with an expressible gene for each of the genes of (a) lacking in

the culture of cells, the gene being operably linked to a promoter.

43. A method of producing a synthetic antigen presenting a cell line comprising:

a) establishing of a culture of cells;

b) transfecting the culture with an expressible Class I MHC heavy chain gene operably linked to a promoter;

c) transfecting the culture with an expressible β-2 microglobulin gene operably linked to a second promoter;

d) transfecting the culture with an expressible costimulatory molecule gene operably linked to a third promoter; and

e) transfecting the culture with an expressible adhesion molecule gene operably linked to a third promoter.

44. A method of producing a synthetic antigen presenting cell line comprising:

a) establishing a culture of cells lacking a gene to at least one of: Class I MHC heavy chain, β-2 microglobulin and co-stimulatory molecule;

b) transfecting the culture with an expressible gene for each of the genes of (a) lacking in the culture of cells, the gene being linked to a first operable promoter; and

c) transfecting the culture with an expressible adhesion molecule gene, the gene being linked to a second operable promoter.

45. A method for activating CD8 · T-cells against a selected peptide *in vitro*, the method comprising:

a) providing the cell line of claim 1;

b) culturing the cell line under conditions such that MHC molecules are produced on the surface of the cell line that are bound to the selected peptide; and

c) contacting the cultured cells with the $CD8^+$ T-cells against the selected peptide.

46. The method of claim 45 wherein the cell line is a poikilotherm.

47. The method of claim 45 further comprising the step of separating the activated $CD8^+$ T-cells from the cell line.

48. The method of claim 47 further comprising the step of adding the activated $CD8^+$ T-cells to an acceptable carrier or excipient to form a suspension.

49. The method of claim 48 further comprising the step of administering the suspension to a patient.

50. A synthetic antigen-presenting matrix comprising:

a) a support;

b) extracellular portion of MHC molecules capable of binding to a selected peptide and being operably linked to the support; and

c) an assisting molecule operably linked to the support such that the extracellular portion of the MHC and assisting molecules are present in sufficient numbers to activate a population of T-cell lymphocytes against the peptide when the peptide is bound to the extracellular portion of the MHC molecule.

51. The matrix of claim 50 wherein the support is a cell fragment.

52. The matrix of claim 50 wherein the support is a cell.

53. The matrix of claim 52 wherein the extracellular portion of the MHC molecule is linked to the cell by a transmembrane domain of an MHC molecule.

53. The matrix of claim 53 wherein the extracellular portion of the MHC molecule is part of a complete MHC molecule.

54. The matrix of claim 50 wherein the support is liposome.

55. The matrix of claim 50 wherein the support is a solid surface.

56. The matrix of claim 50 wherein the extracellular portion of the MHC molecule is linked to an epitope which reacts with an antibody to link the portion to the support.

57. The matrix of claim 50 wherein the extracellular portion of the MHC molecule is linked to $(His)_6$ which reacts with nickel to link the portion to the support.

58. The matrix of claim 50 wherein the support is a porous material.

59. The matrix of claim 50 wherein the assisting molecule is a costimulatory molecule.

60. The matrix of claim 59 wherein the costimulatory molecule is B7.1 or B7.2.

61. The matrix of claim 59 wherein the costimulatory molecule is is at least a portion of an anti-CD80 antibody.

62. The matrix of claim 50 wherein the assisting molecule is an adhesion molecule.

63. The matrix of claim 62 wherein the adhesion molecule is ICAM-1, ICAM-2, ICAM-3 or LFA-3.

64. The matrix of claim 50 having two assisting molecules, the first assisting molecule being a costimulatory molecule and the second assisting molecule being an adhesion molecule.

65. The matrix of claim 50 wherein the extracellular portion of the MHC molecule is empty.

66. The matrix of claim 50 wherein the peptide is bound to the extracellular portion of the MHC molecule.

67. The matrix of claim 64 wherein the peptide is bound to the extracellular portion of the MHC molecule.

68. A synthetic antigen-presenting matrix comprising:

a) a support;

b) extracellular portion of MHC molecules capable of binding to a selected peptide and being operably linked to the support;

c) B-7.1 or B-7.2 molecules or a combination thereof operably linked to the support; and

d) ICAM-1 molecules operably linked to the support such that the molecules are present in sufficient amount to activate a population of T-cell lymphocytes against the peptide when the peptide is bound to the extracellular portion of the MHC molecule.

69. A method of producing a synthetic T-cell lymphocyte antigen activating matrix comprising:

a) establishing a first culture of cells;

b) transfecting the culture with an expressible Class I MHC heavy chain gene for at least the extracellular portion of the heavy chain operably linked to a promoter;

c) transfecting the culture with an expressible β-2 microglobulin gene operably linked to a second promoter;

d) harvesting the produced MHC molecule portions;

e) establishing a second culture of cells;

f) transfecting the second culture with an expressible assisting molecule gene for at least the

MISSING PAGE

b) contacting the matrix cells with the $CD8^+$ T-cells.

79. A method for generating cytotoxic $CD8^+$ T-cells against a selected peptide, the method comprising:

a) providing the matrix of claim 67;

b) contacting naive $CD8^+$ T-cells with the matrix *in vitro*.

80. The matrix of claim 79 wherein the support is a cell.

81. The method of claim 79 wherein the costimulatory molecule is B7.1 or B7.2.

82. The method of claim 79 wherein the costimulatory molecule is at least a portion of an anti-CD28 antibody.

83. The method of claim 79 wherein the adhesion molecule is ICAM-1, ICAM-2, ICAM-3 or LFA-3.

84. A method of treating a tumor in a patient comprising:

a) identifying a tumor specific antigen;

b) collecting $CD8^+$ T-cells from the patient;

c) contacting the $CD8^+$ T-cells with the matrix of claim 67 *in vitro* in a sufficient amount and for a sufficient time to generate cytotoxic $CD8^+$ T-cells; and

d) returning the cytotoxic $CD8^+$ T-cells to the patient.

85. The method of claim 84 wherein the antigen is a self antigen of the patient.

86. The method of claim 85 wherein the assisting molecule is B7.1 or B7.2 and the adhesion molecule is ICAM-1, ICAM-2 or ICAM-3.

87. The method of claim 84 wherein the assisting molecule is B7.1 and the adhesion molecule is ICAM-1.

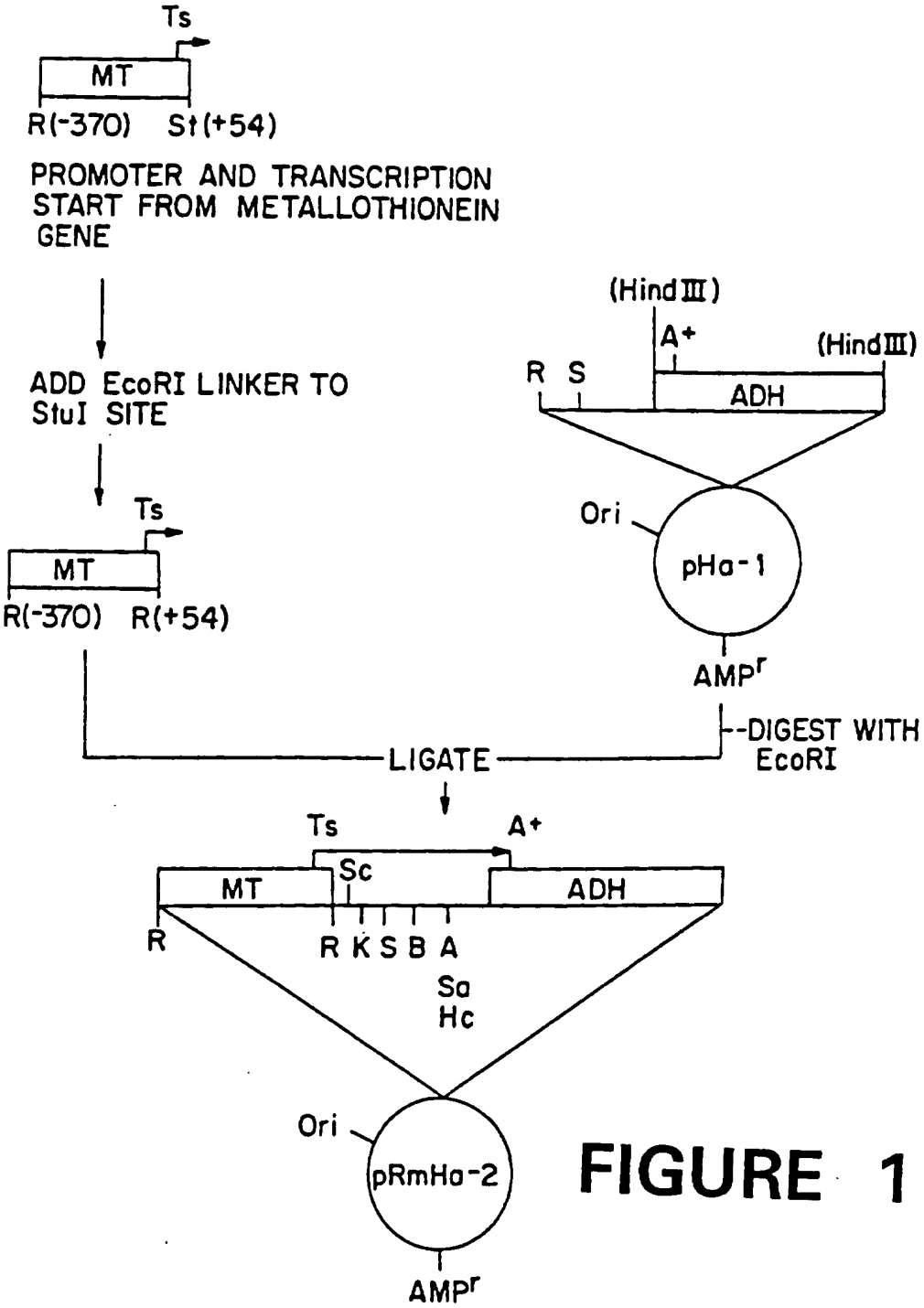
Ts
MT
R(-370) St(+54)
PROMOTER AND TRANSCRIPTION START FROM METALLOTHIONEIN GENE
ADD EcoRI LINKER TO StuI SITE
Ts
MT
R(-370) R(+54)
(HindIII)
A+
(HindIII)
R S
ADH
Ori
pHa-1
AMPr
LIGATE
--DIGEST WITH EcoRI
Ts
A+
Sc
MT
ADH
R
R K S B A
Sa
Hc
Ori
pRmHa-2
AMPr

FIGURE 1

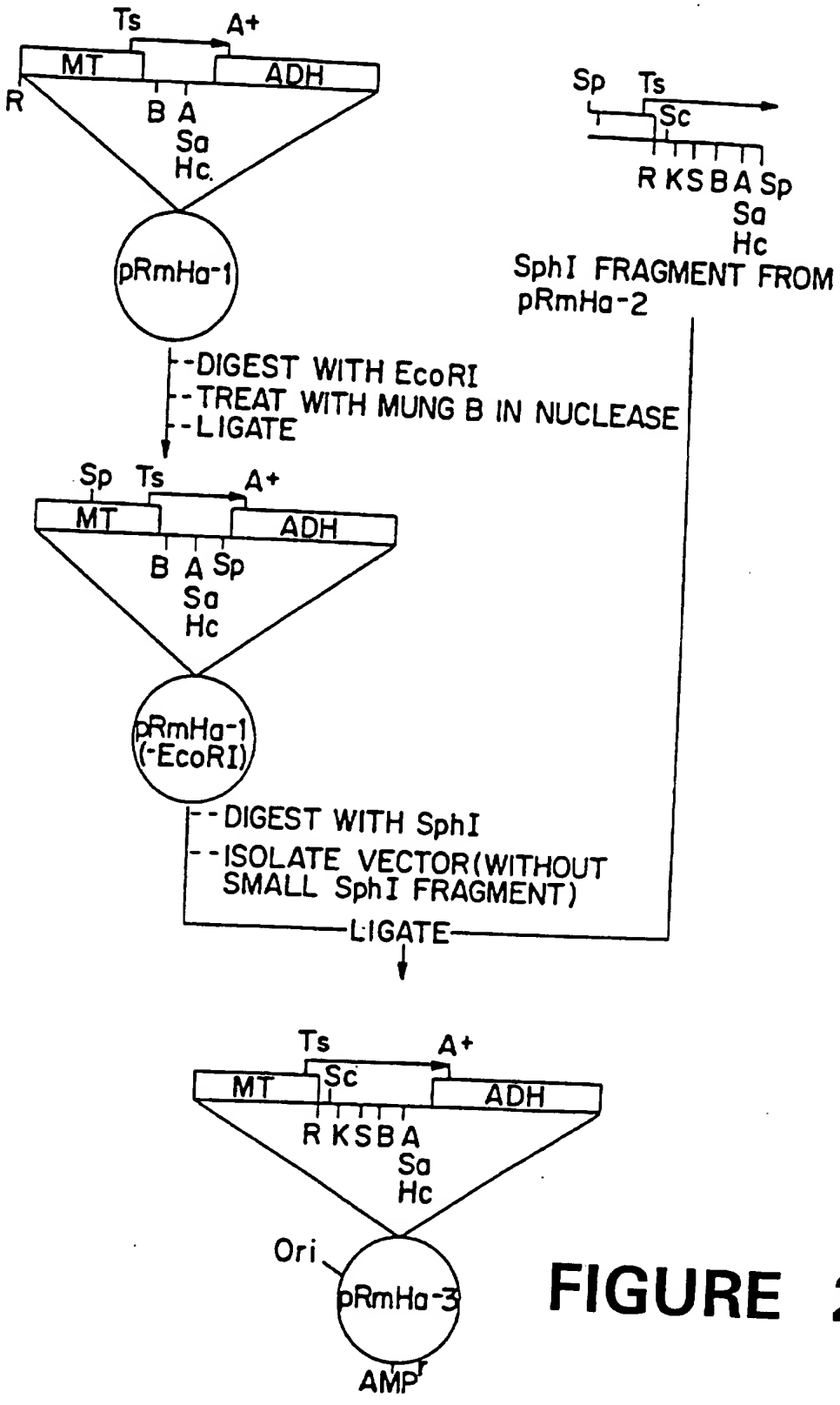
Ts
A+
MT
ADH
R
B A
Sa
Hc
pRmHa-1
Sp Ts
Sc
R K S B A Sp
Sa
Hc
SphI FRAGMENT FROM
pRmHa-2
-DIGEST WITH EcoRI
-TREAT WITH MUNG B IN NUCLEASE
-LIGATE
Sp Ts
A+
MT
ADH
B A Sp
Sa
Hc
pRmHa-1
(-EcoRI)
--DIGEST WITH SphI
--ISOLATE VECTOR(WITHOUT
SMALL SphI FRAGMENT)
LIGATE
Ts
A+
MT
Sc
ADH
R K S B A
Sa
Hc
Ori
pRmHa-3
AMP^r

FIGURE 2

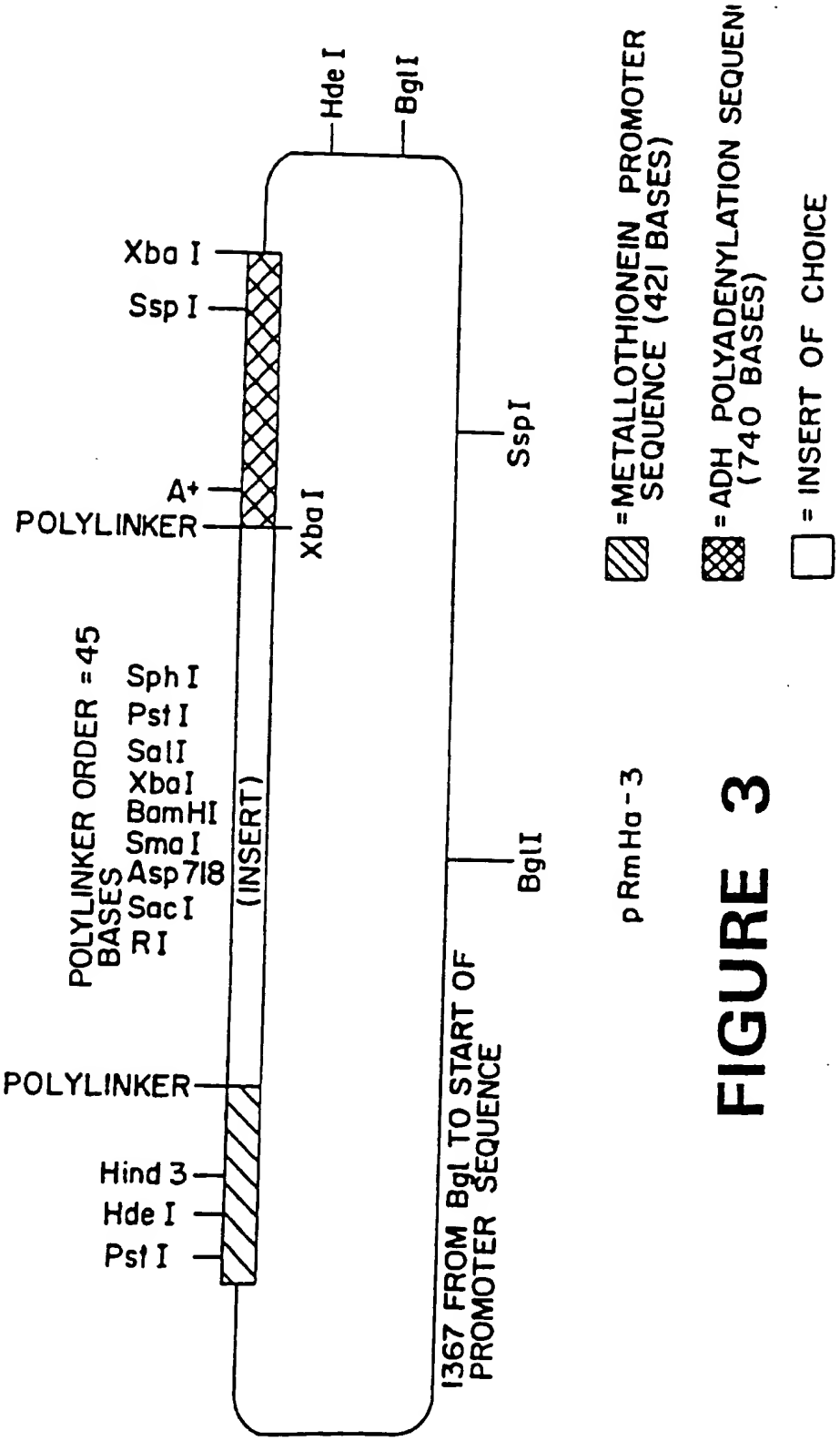
Hde I
BglI
Xba I
Ssp I
SspI
A+
POLYLINKER
XbaI
POLYLINKER ORDER = 45 BASES
Sph I
Pst I
Sal I
Xba I
BamHI
Sma I
Asp 718
Sac I
RI
(INSERT)
BglI
POLYLINKER
Hind 3
Hde I
Pst I
1367 FROM Bgl TO START OF PROMOTER SEQUENCE
= METALLOTHIONEIN PROMOTER SEQUENCE (421 BASES)
= ADH POLYADENYLATION SEQUEN (740 BASES)
= INSERT OF CHOICE
pRmHa-3

FIGURE 3

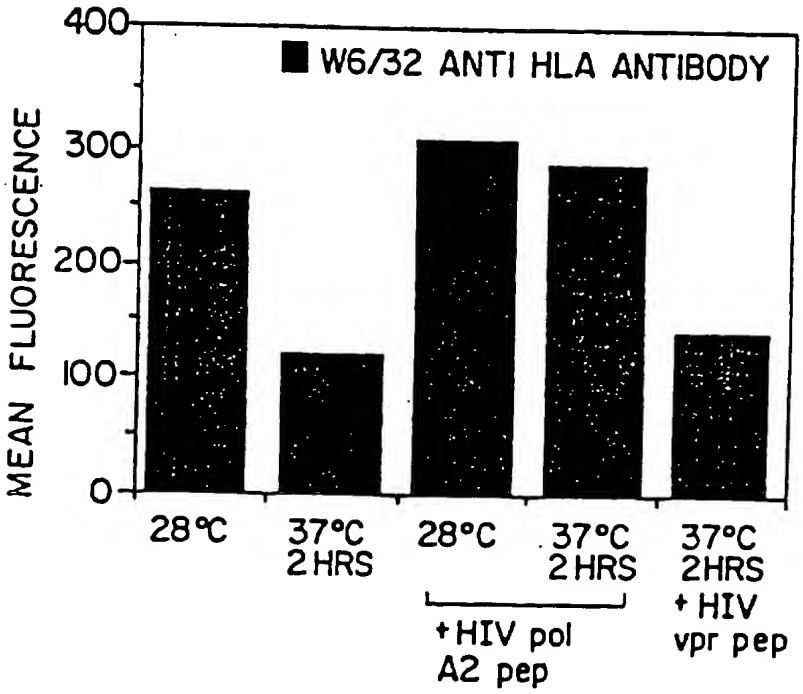
400
300
200
100
0
MEAN FLUORESCENCE
W6/32 ANTI HLA ANTIBODY
28°C
37°C 2HRS
28°C
37°C 2HRS
+HIV pol A2 pep
37°C 2HRS + HIV vpr pep

FIGURE 4

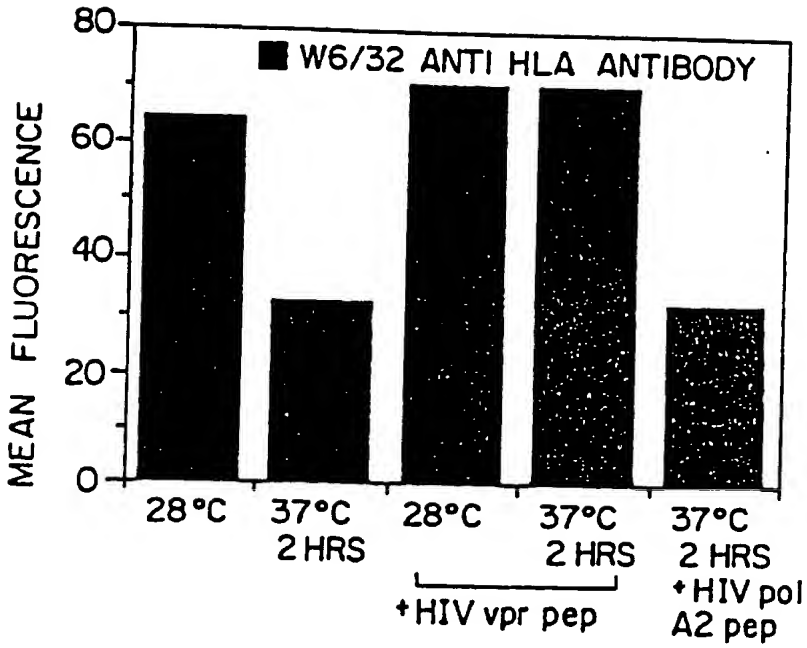
80
60
40
20
0
MEAN FLUORESCENCE
W6/32 ANTI HLA ANTIBODY
28°C
37°C 2 HRS
28°C
37°C 2 HRS
+HIV vpr pep
37°C 2 HRS +HIV pol A2 pep

FIGURE 5

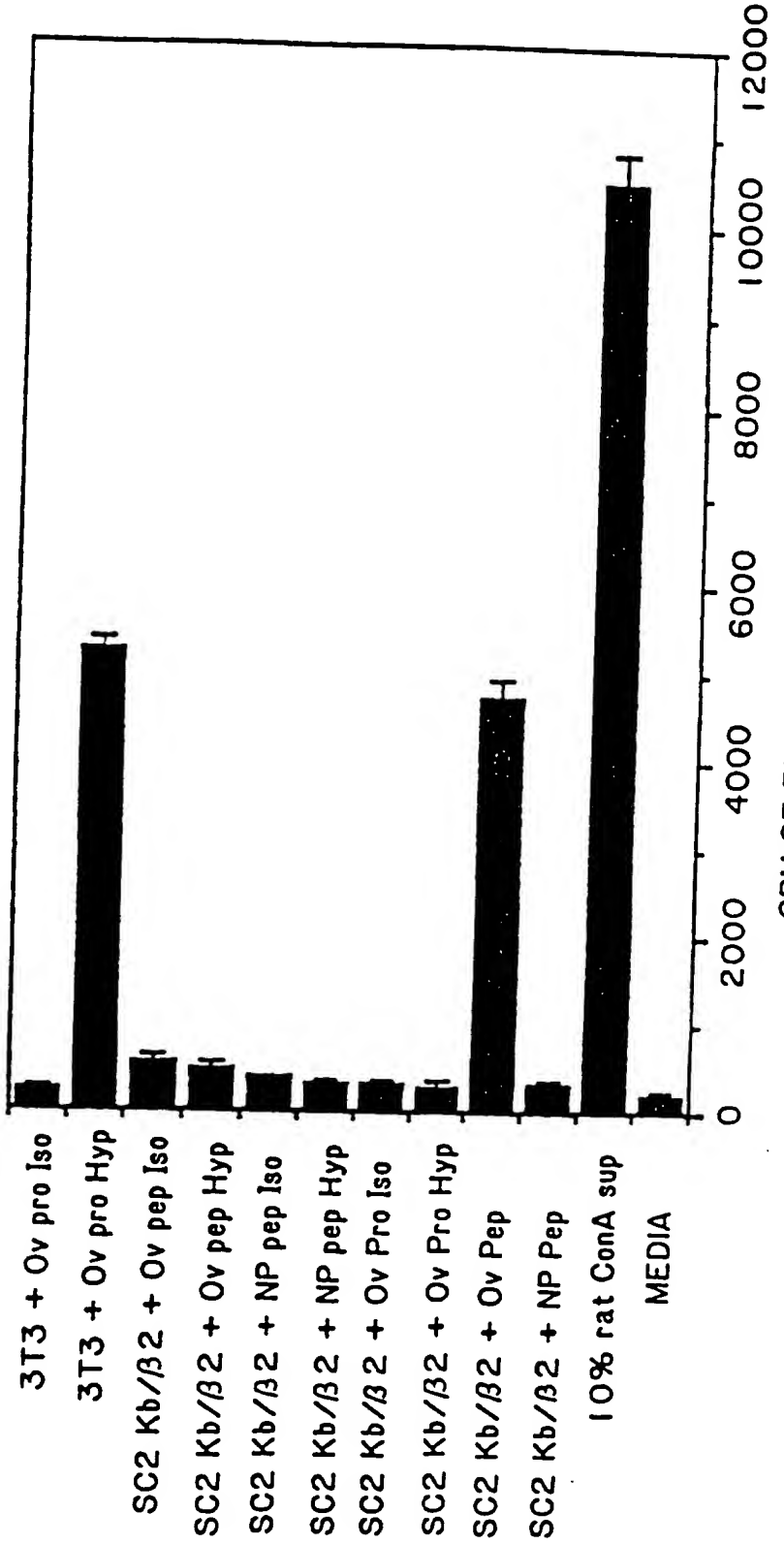
3T3 + Ov pro Iso
3T3 + Ov pro Hyp
SC2 Kb/β2 + Ov pep Iso
SC2 Kb/β2 + Ov pep Hyp
SC2 Kb/β2 + NP pep Iso
SC2 Kb/β2 + NP pep Hyp
SC2 Kb/β2 + Ov Pro Iso
SC2 Kb/β2 + Ov Pro Hyp
SC2 Kb/β2 + Ov Pep
SC2 Kb/β2 + NP Pep
10% rat ConA sup
MEDIA
0
2000
4000
6000
8000
10000
12000
CPM OF 3H THIAMIDINE INCORPORATED

FIGURE 6

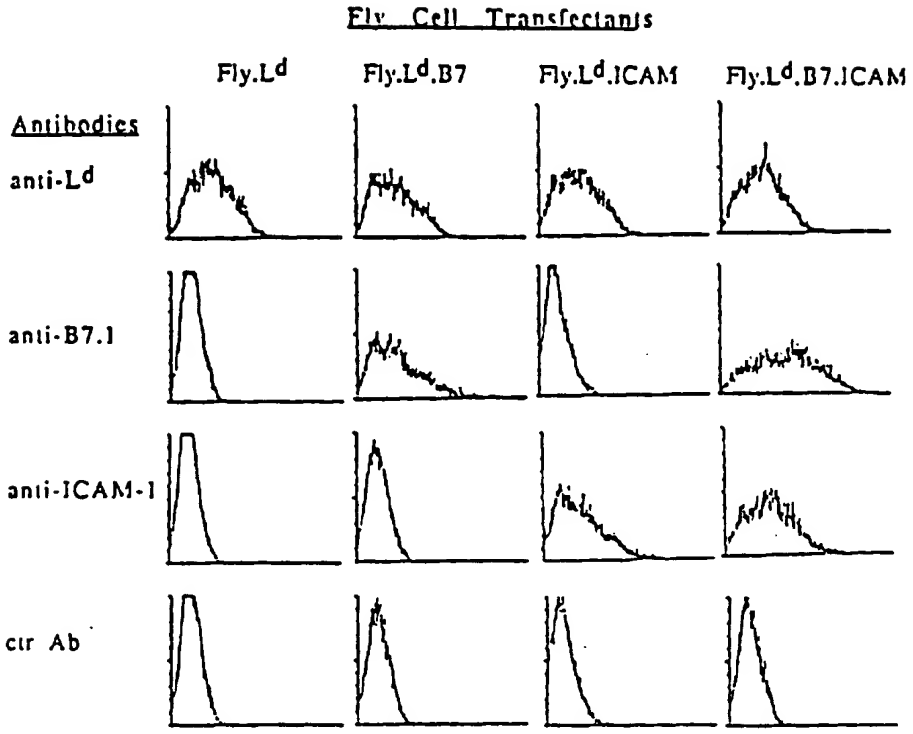
Fly Cell Transfectants
Fly.Ld
Fly.Ld.B7
Fly.Ld.ICAM
Fly.Ld.B7.ICAM
Antibodies
anti-Ld
anti-B7.1
anti-ICAM-1
ctr Ab

FIGURE 7

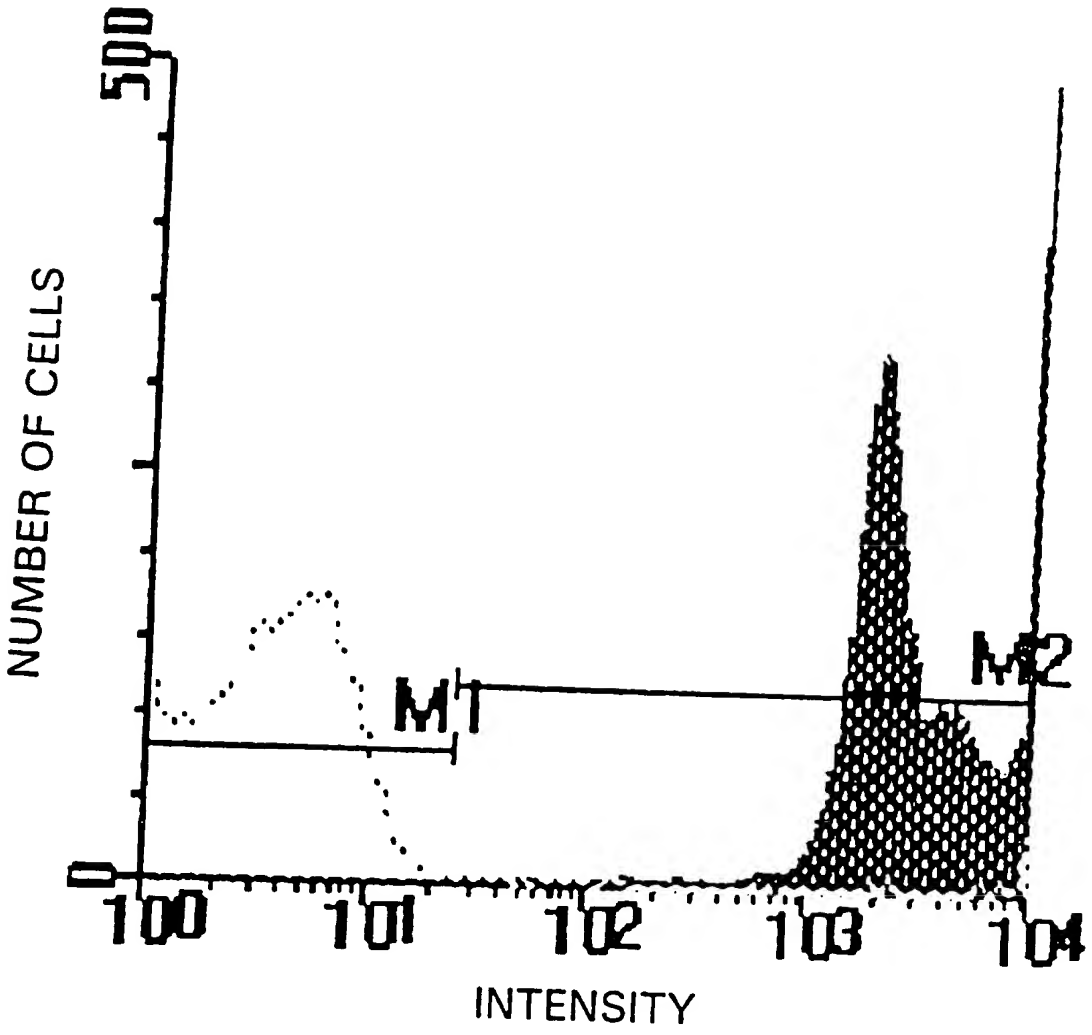

FIGURE 8

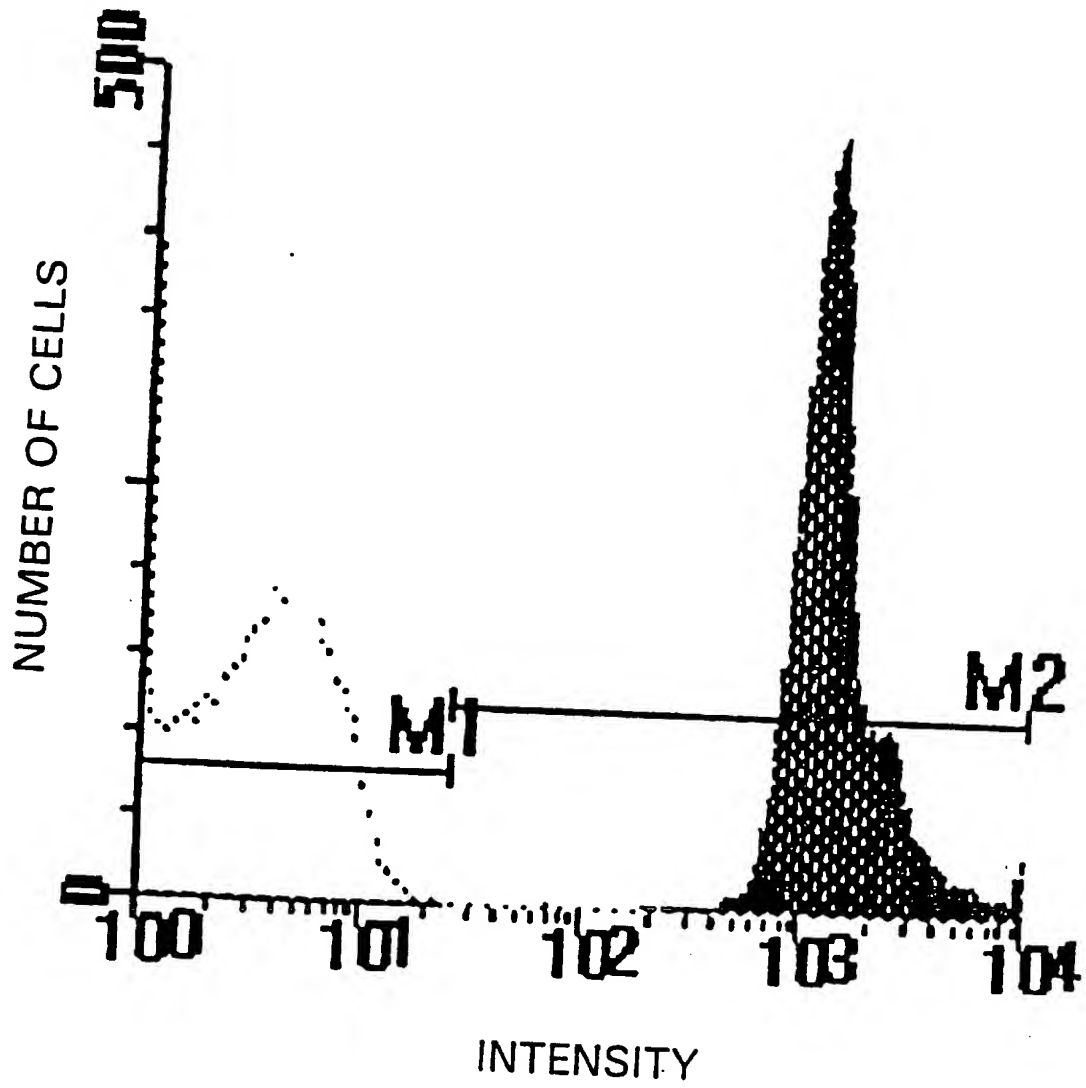

FIGURE 9

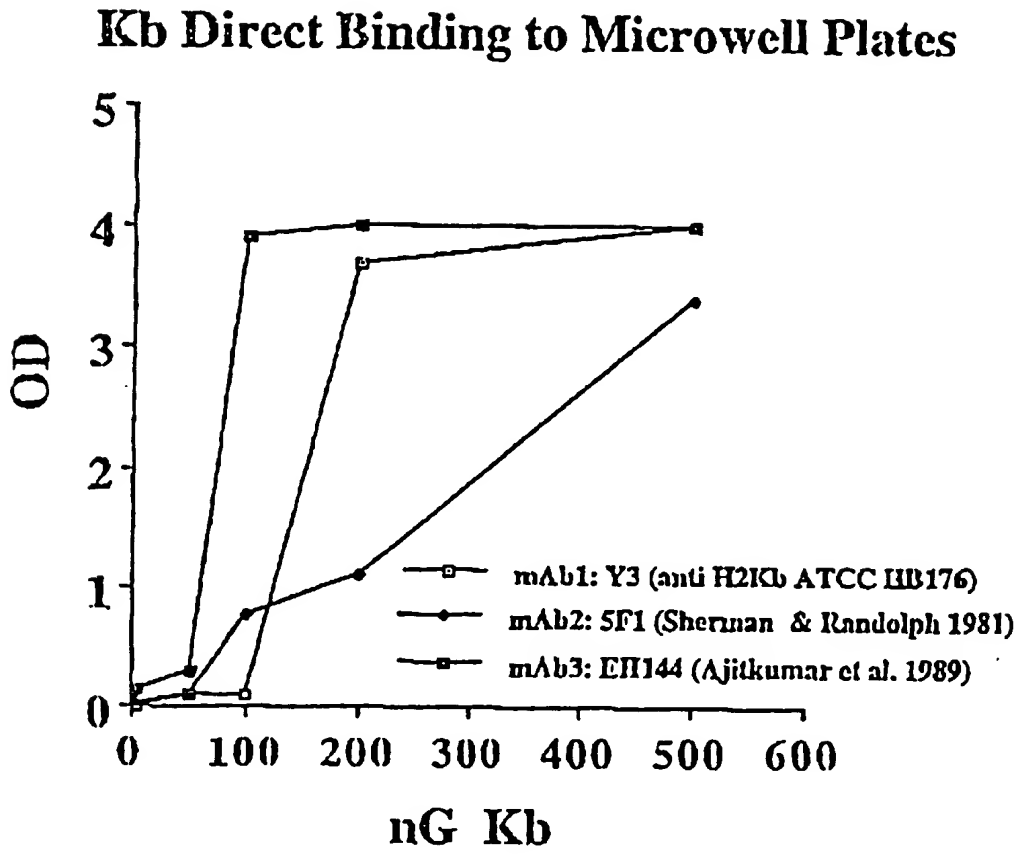
Kb Direct Binding to Microwell Plates
OD
5
4
3
2
1
0
0
100
200
300
400
500
600
nG Kb
mAb1: Y3 (anti H2Kb ATCC HB176)
mAb2: 5F1 (Sherman & Randolph 1981)
mAb3: EH144 (Ajitkumar et al. 1989)

FIGURE 10

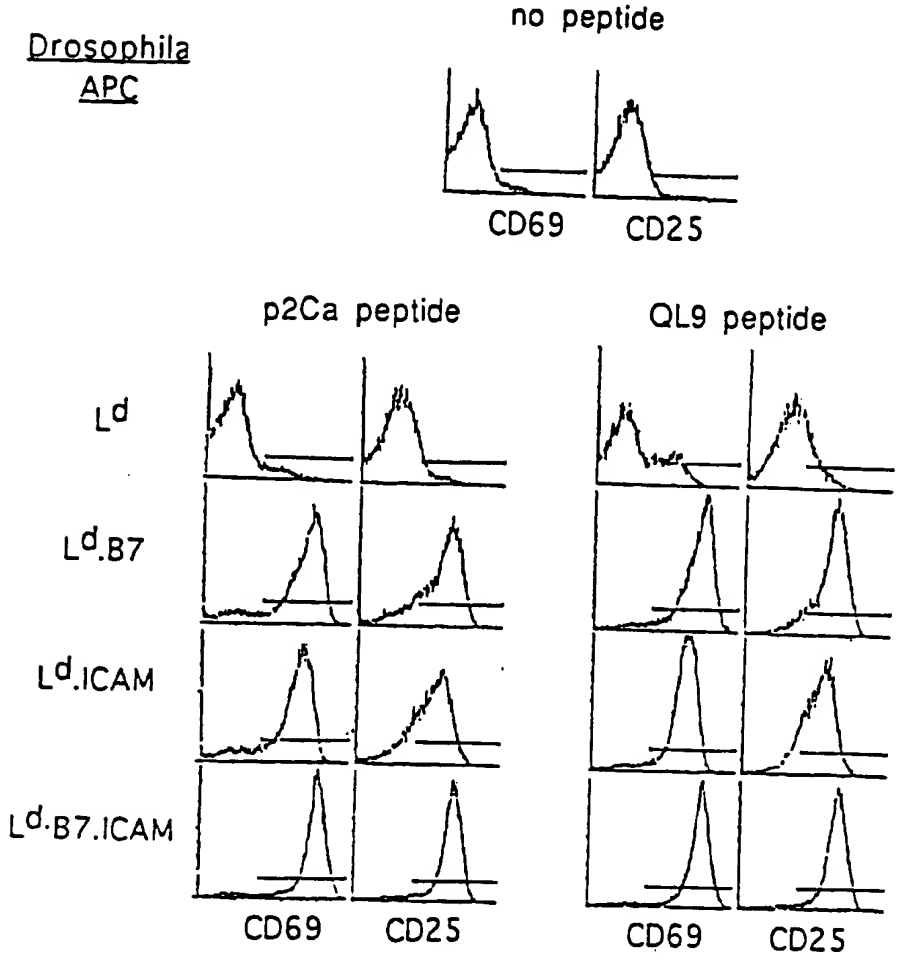
Drosophila APC
no peptide
CD69
CD25
p2Ca peptide
QL9 peptide
Ld
Ld.B7
Ld.ICAM
Ld.B7.ICAM
CD69
CD25
CD69
CD25

FIGURE 11

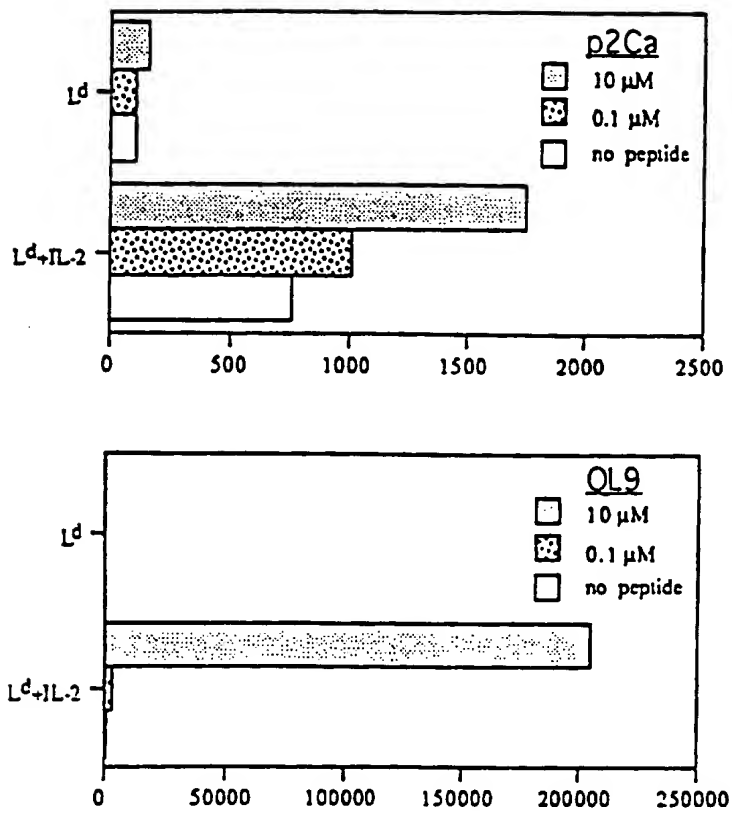
p2Ca
10 µM
0.1 µM
no peptide
Ld
Ld+IL-2
0
500
1000
1500
2000
2500
QL9
10 µM
0.1 µM
no peptide
Ld
Ld+IL-2
0
50000
100000
150000
200000
250000
[3H] TdR Incorporation (cpm)

FIGURE 12

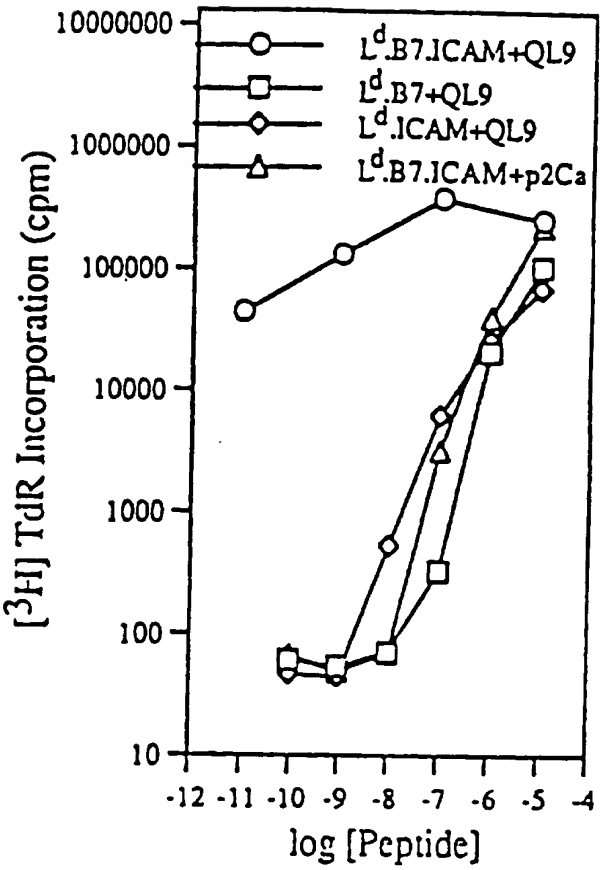
10000000
1000000
100000
10000
1000
100
10
[3H] TdR Incorporation (cpm)
-12 -11 -10 -9 -8 -7 -6 -5 -4
log [Peptide]
Ld.B7.ICAM+QL9
Ld.B7+QL9
Ld.ICAM+QL9
Ld.B7.ICAM+p2Ca

FIGURE 13

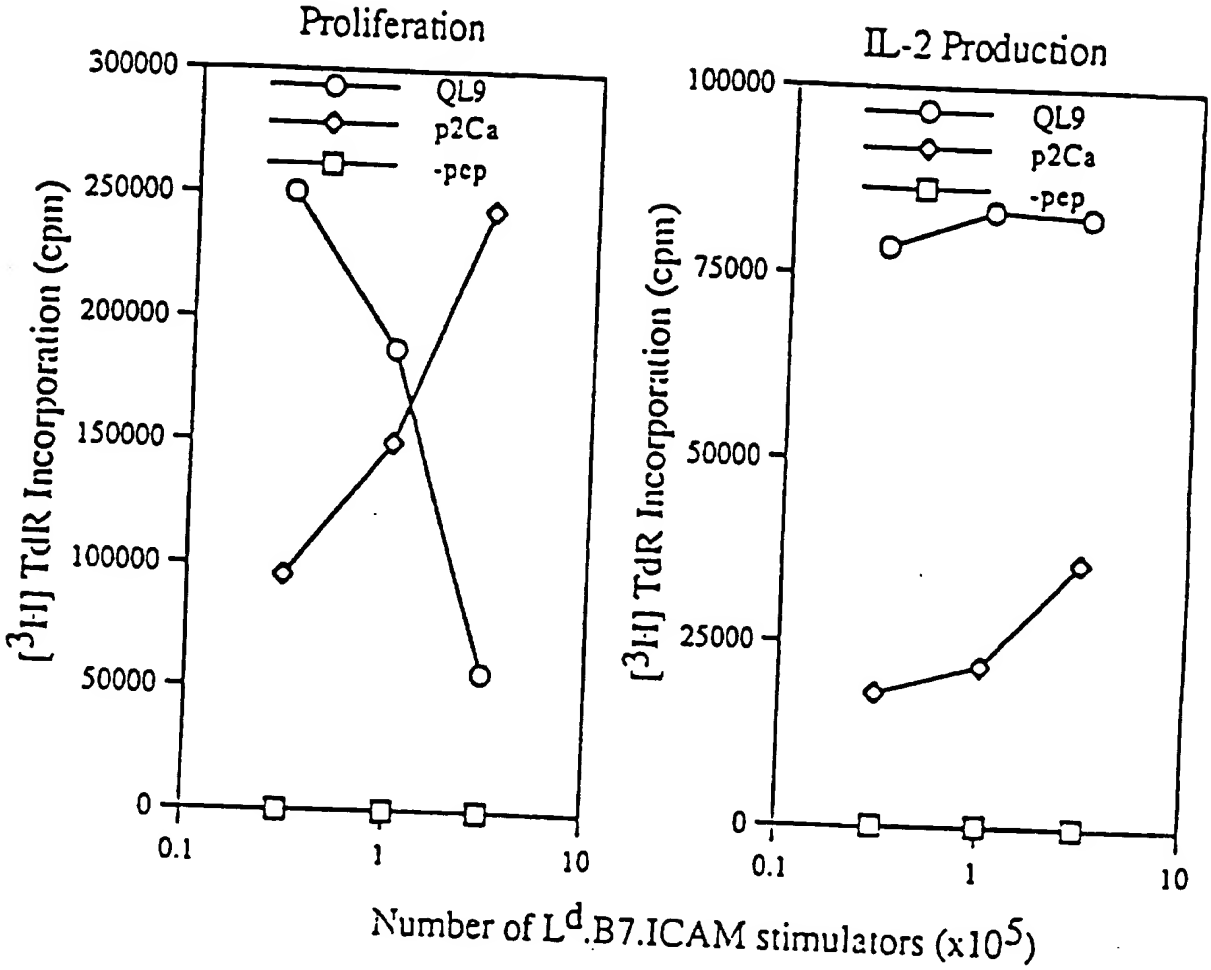
Proliferation
IL-2 Production
QL9
p2Ca
-pep
[3H] TdR Incorporation (cpm)
300000
250000
200000
150000
100000
50000
0
100000
75000
50000
25000
0
0.1
1
10
Number of Ld.B7.ICAM stimulators (x10^5)

FIGURE 14

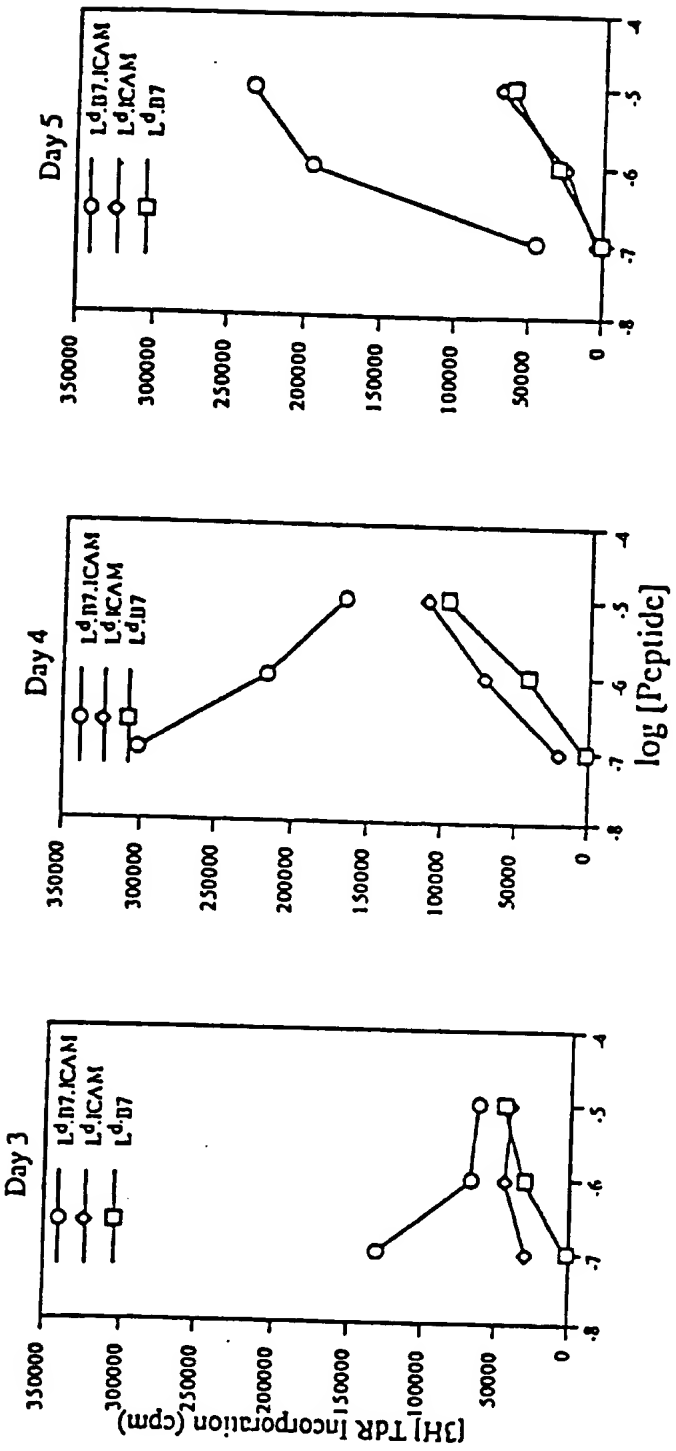
Day 3
Day 4
Day 5
L^d.B7.ICAM
L^d.ICAM
L^d.B7
[3H] TdR Incorporation (cpm)
log [Peptide]
350000
300000
250000
200000
150000
100000
50000
0
-4
-5
-6
-7
-8

FIGURE 15

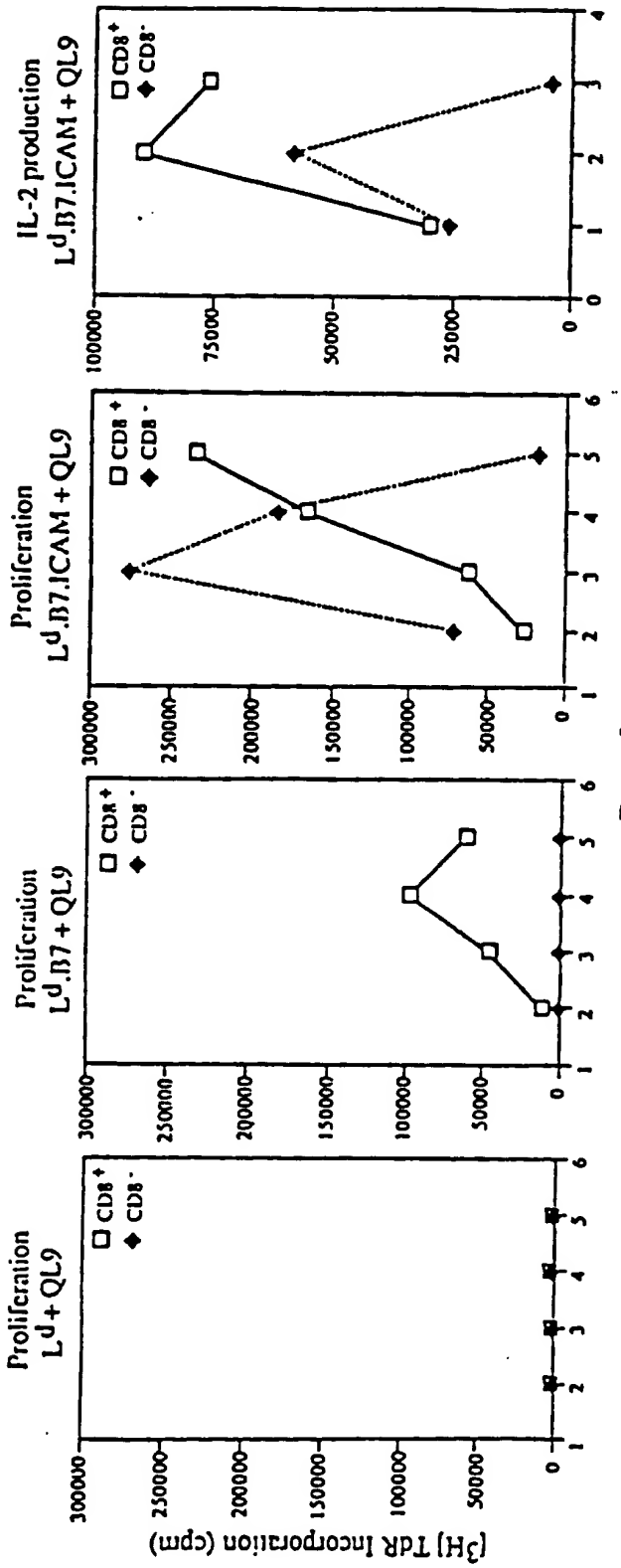
Proliferation
L^d + QL9
Proliferation
L^d.B7 + QL9
Proliferation
L^d.B7.ICAM + QL9
IL-2 production
L^d.B7.ICAM + QL9
CD8+
CD8-
[3H] TdR Incorporation (cpm)
Day of assay

FIGURE 16

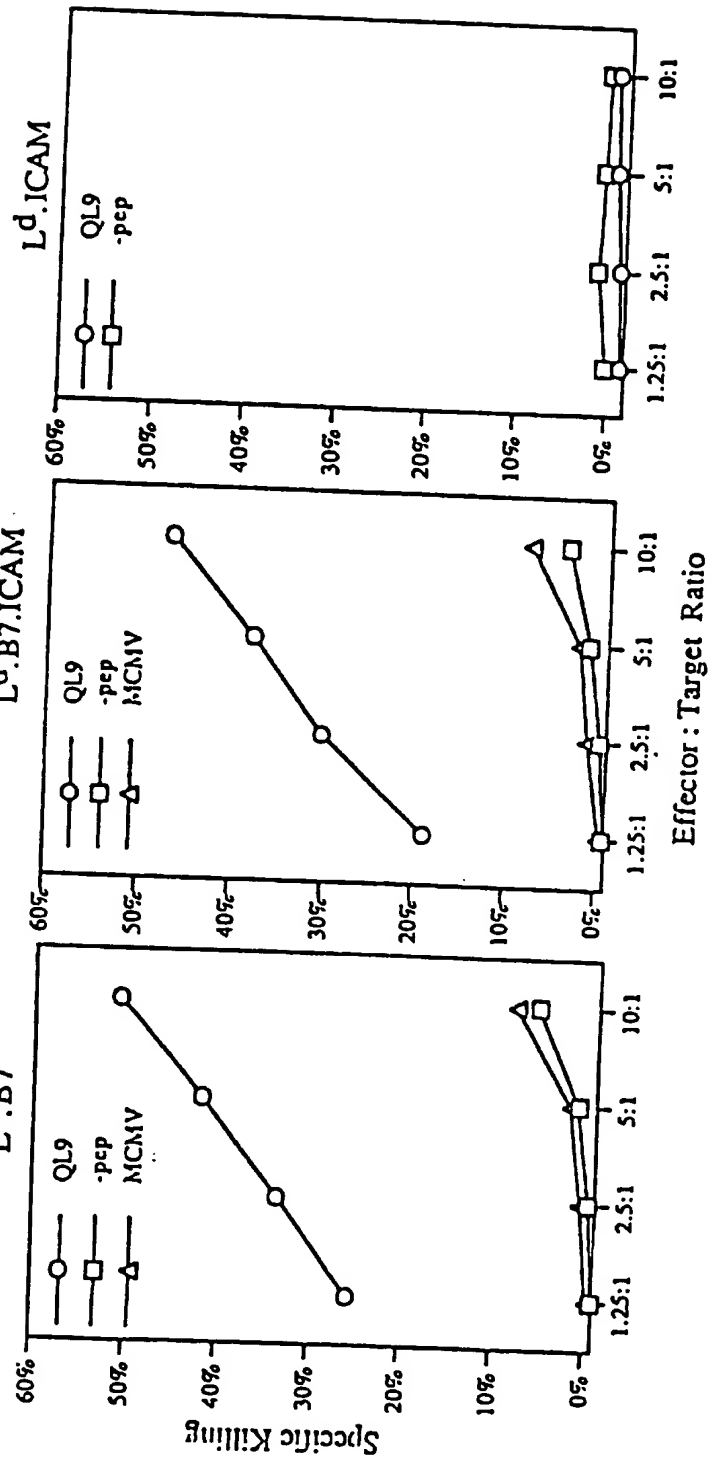
L^d.B7
L^d.B7.ICAM
L^d.ICAM
QL9
-pep
MCMV
Specific Killing
Effector : Target Ratio
1.25:1
2.5:1
5:1
10:1
0%
10%
20%
30%
40%
50%
60%

FIGURE 17

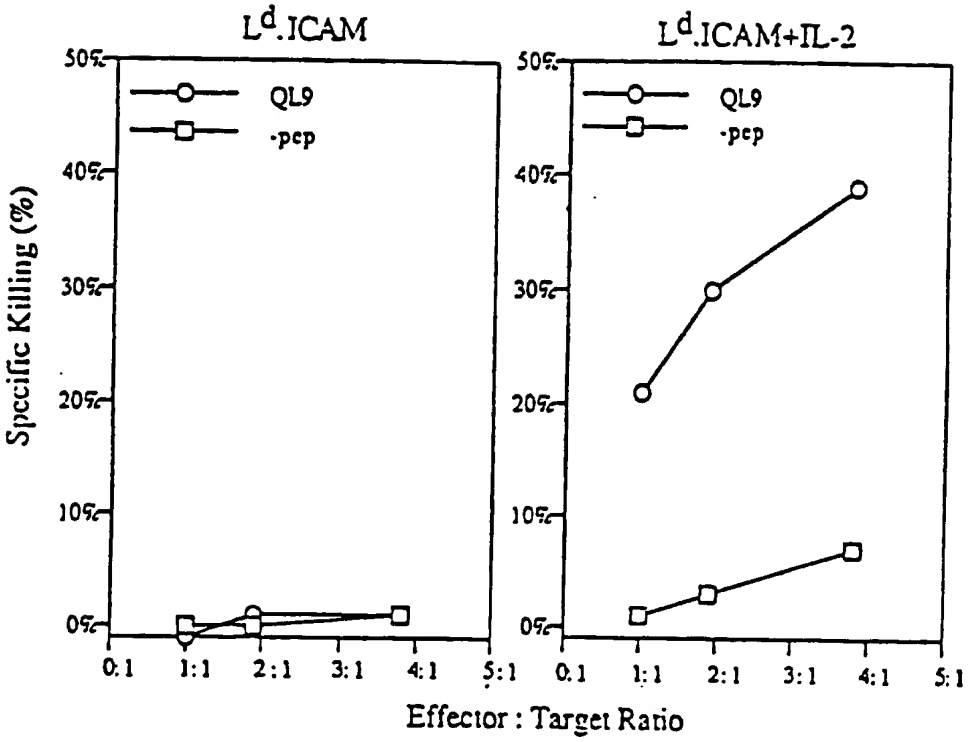
L^d.ICAM
L^d.ICAM+IL-2
QL9
-pep
Specific Killing (%)
50%
40%
30%
20%
10%
0%
0:1
1:1
2:1
3:1
4:1
5:1
Effector : Target Ratio

FIGURE 18

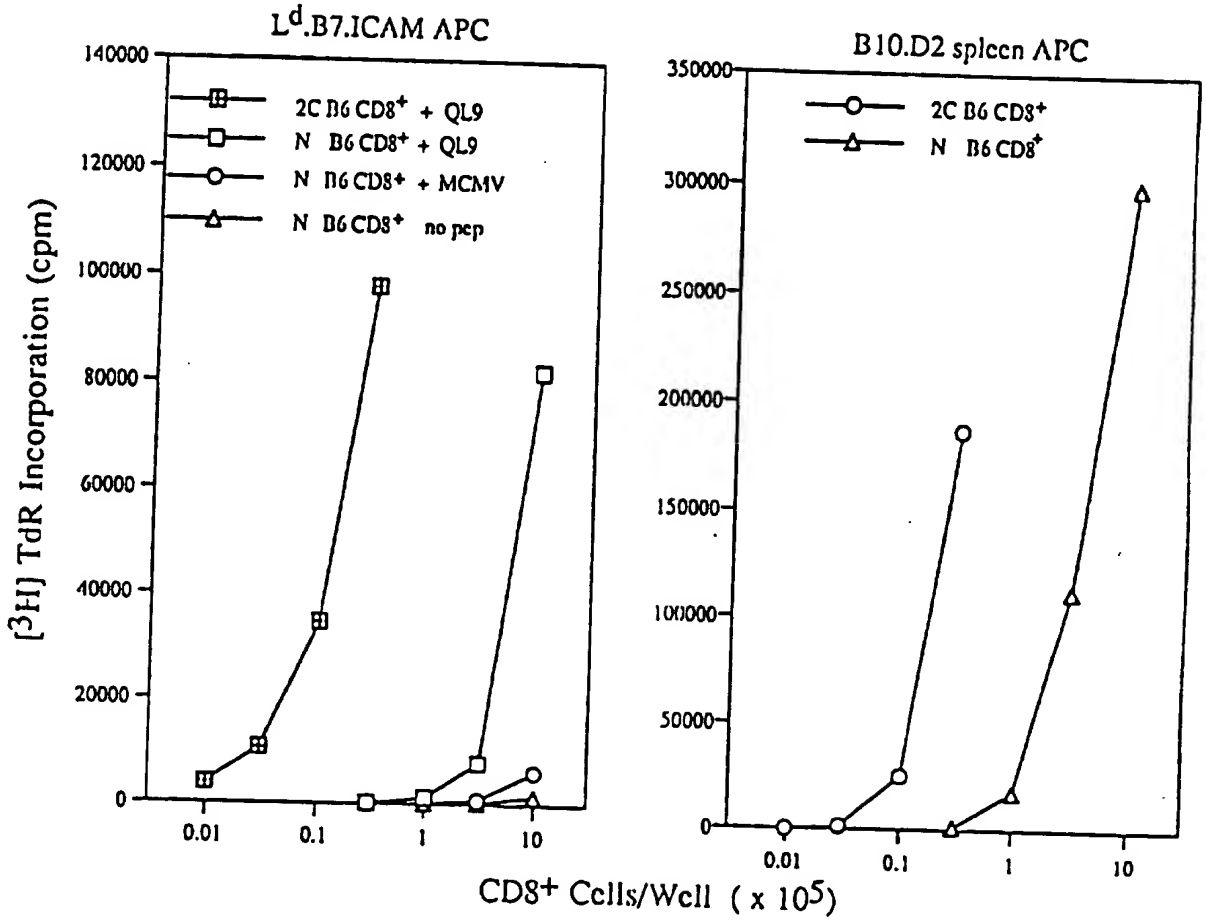
L^d.B7.ICAM APC
B10.D2 spleen APC
2C B6 CD8$^+$ + QL9
N B6 CD8$^+$ + QL9
N B6 CD8$^+$ + MCMV
N B6 CD8$^+$ no pep
2C B6 CD8$^+$
N B6 CD8$^+$
[^{3}H] TdR Incorporation (cpm)
CD8$^+$ Cells/Well (x 10^5)
140000
120000
100000
80000
60000
40000
20000
0
350000
300000
250000
200000
150000
100000
50000
0.01
0.1
1
10

FIGURE 19

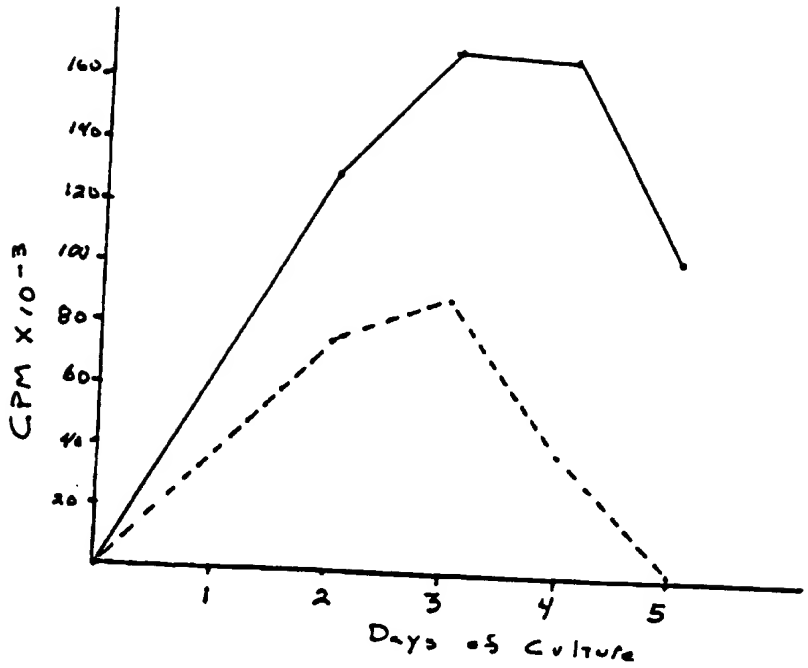

FIGURE 20

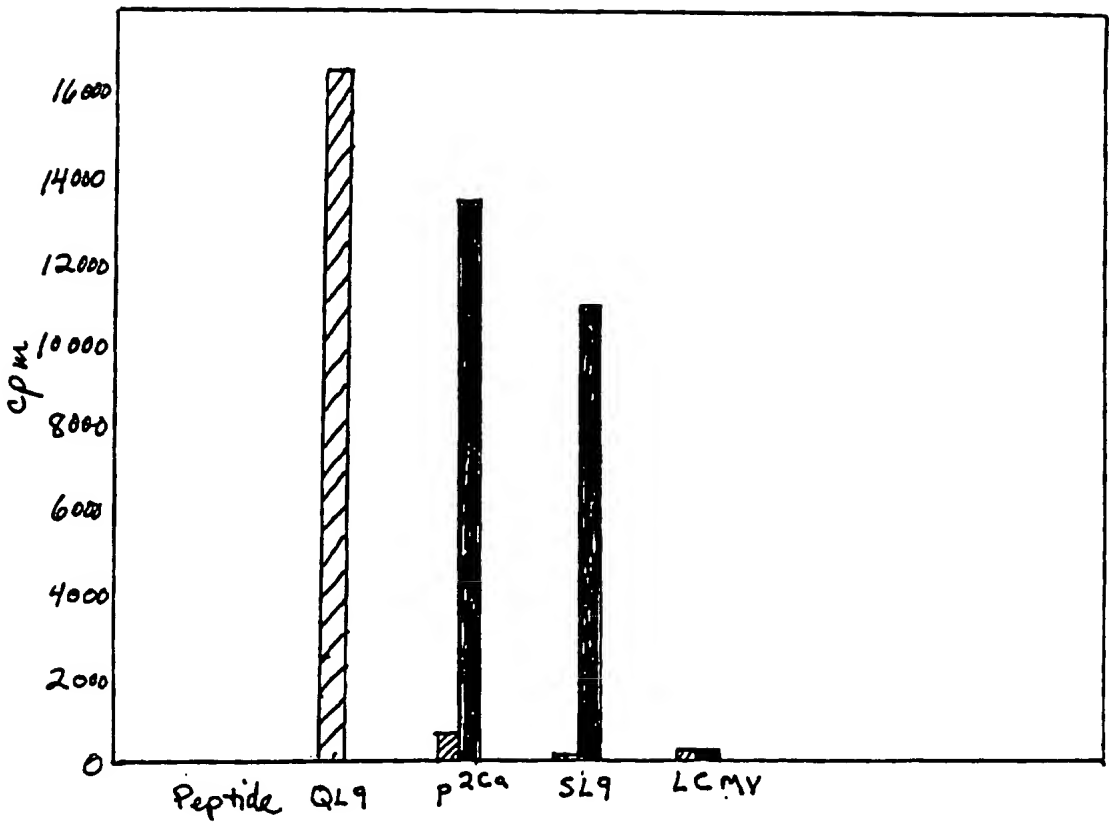
16000
14000
12000
10000
8000
6000
4000
2000
0
cpm
Peptide
QL9
p2Ca
SL9
LCMV

FIGURE 21

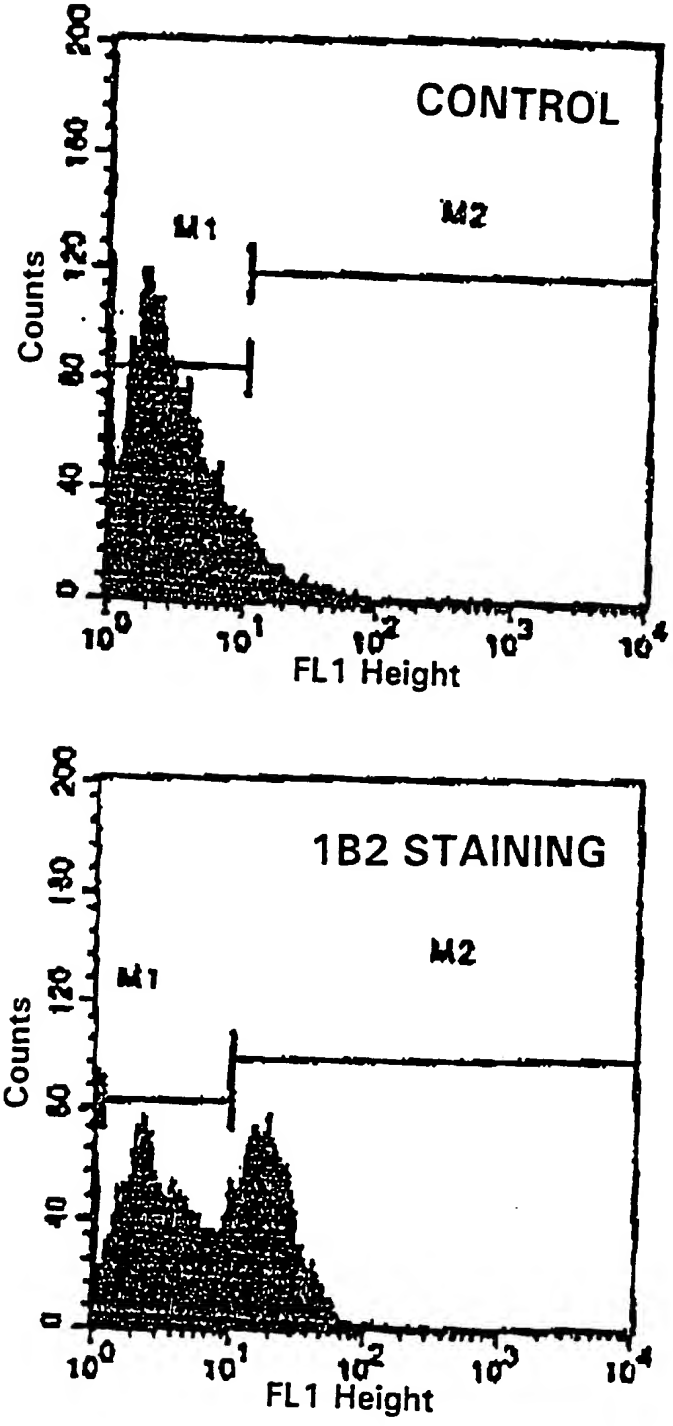
CONTROL
M1
M2
Counts
FL1 Height
1B2 STAINING
M1
M2
Counts
FL1 Height

FIGURE 22

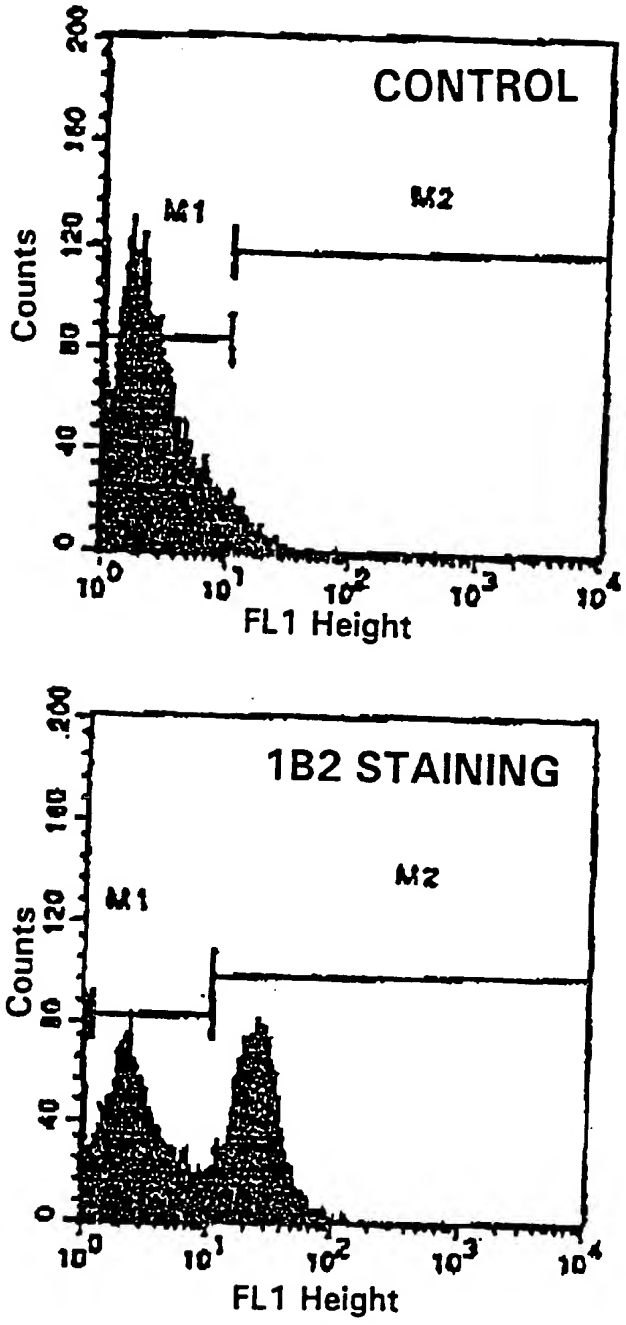
CONTROL
M1
M2
Counts
FL1 Height
1B2 STAINING
M1
M2
Counts
FL1 Height

FIGURE 23

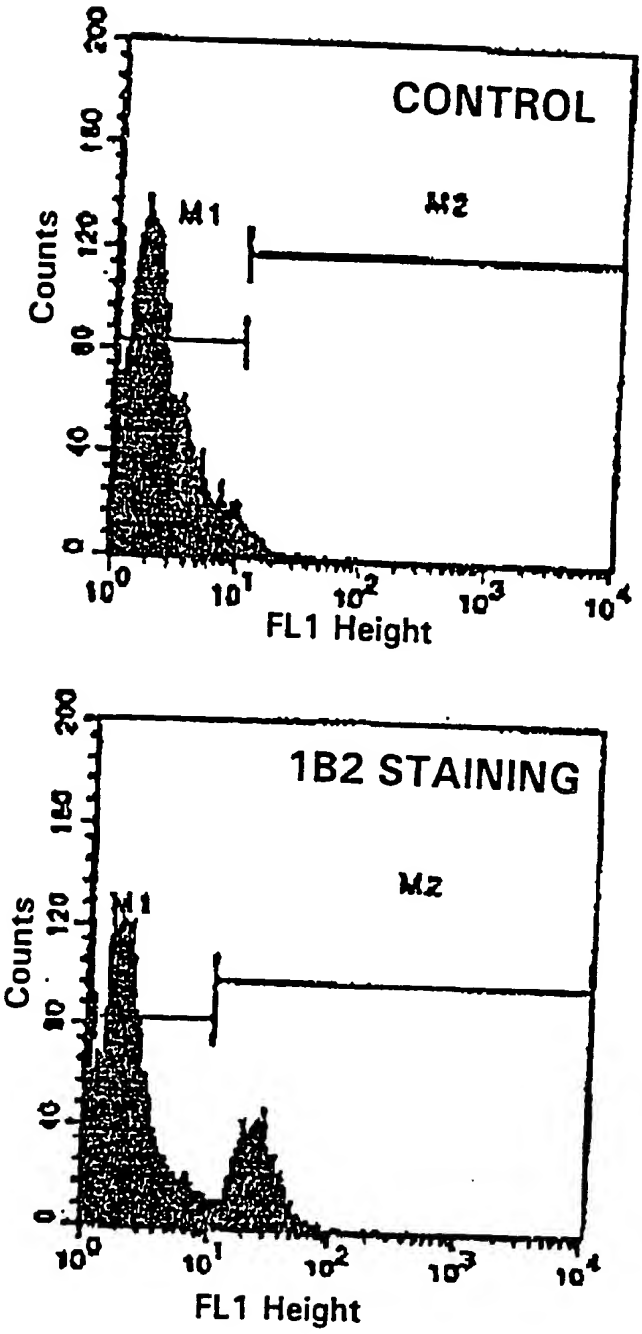
CONTROL
M1
M2
Counts
FL1 Height
1B2 STAINING
M1
M2
Counts
FL1 Height

FIGURE 24

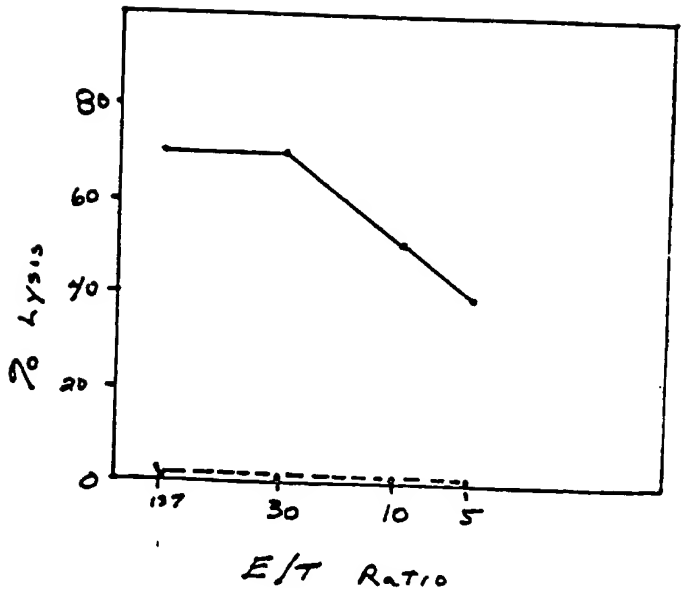

FIGURE 25

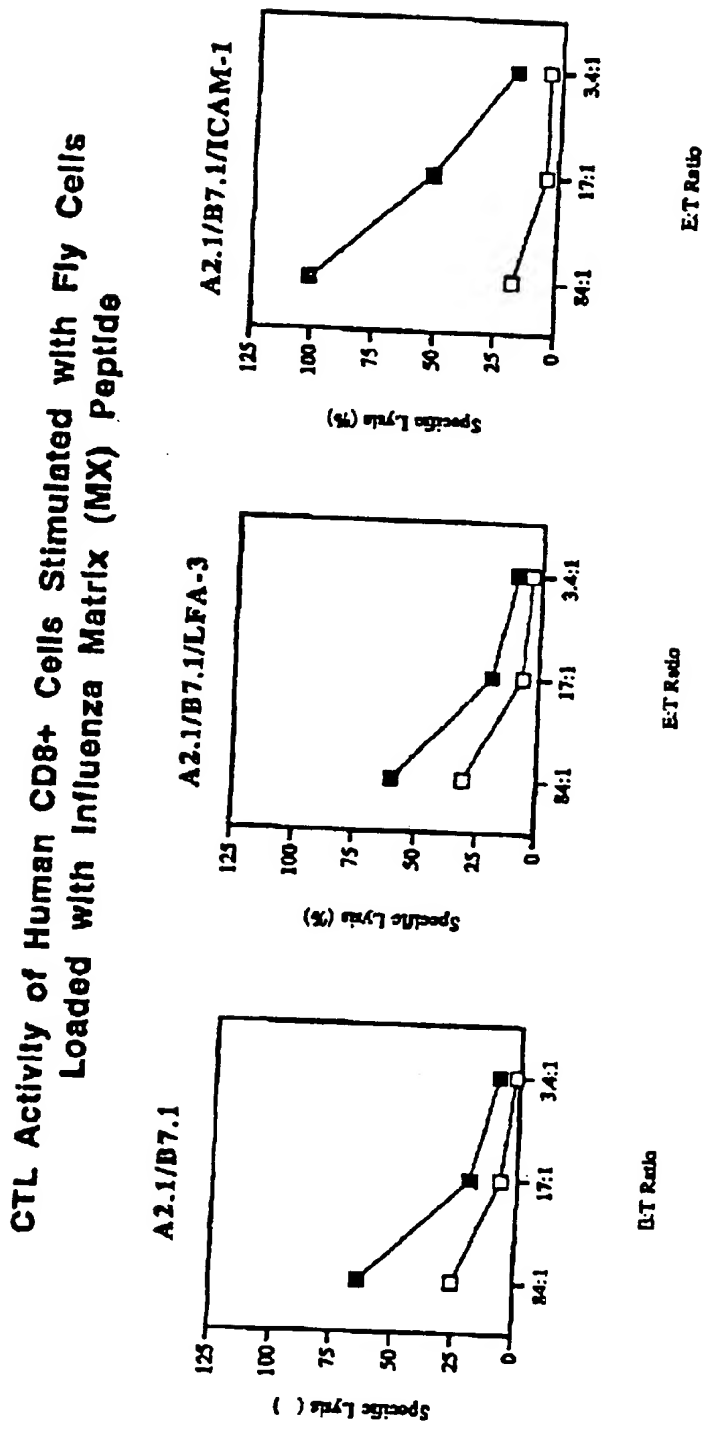
CTL Activity of Human CD8+ Cells Stimulated with Fly Cells Loaded with Influenza Matrix (MX) Peptide
A2.1/B7.1
A2.1/B7.1/LFA-3
A2.1/B7.1/ICAM-1
Specific Lysis ( )
Specific Lysis (%)
125
100
75
50
25
0
84:1
17:1
3.4:1
E:T Ratio
Target Cells: JY+Control Peptide
Target Cells: JY+MX

FIGURE 26

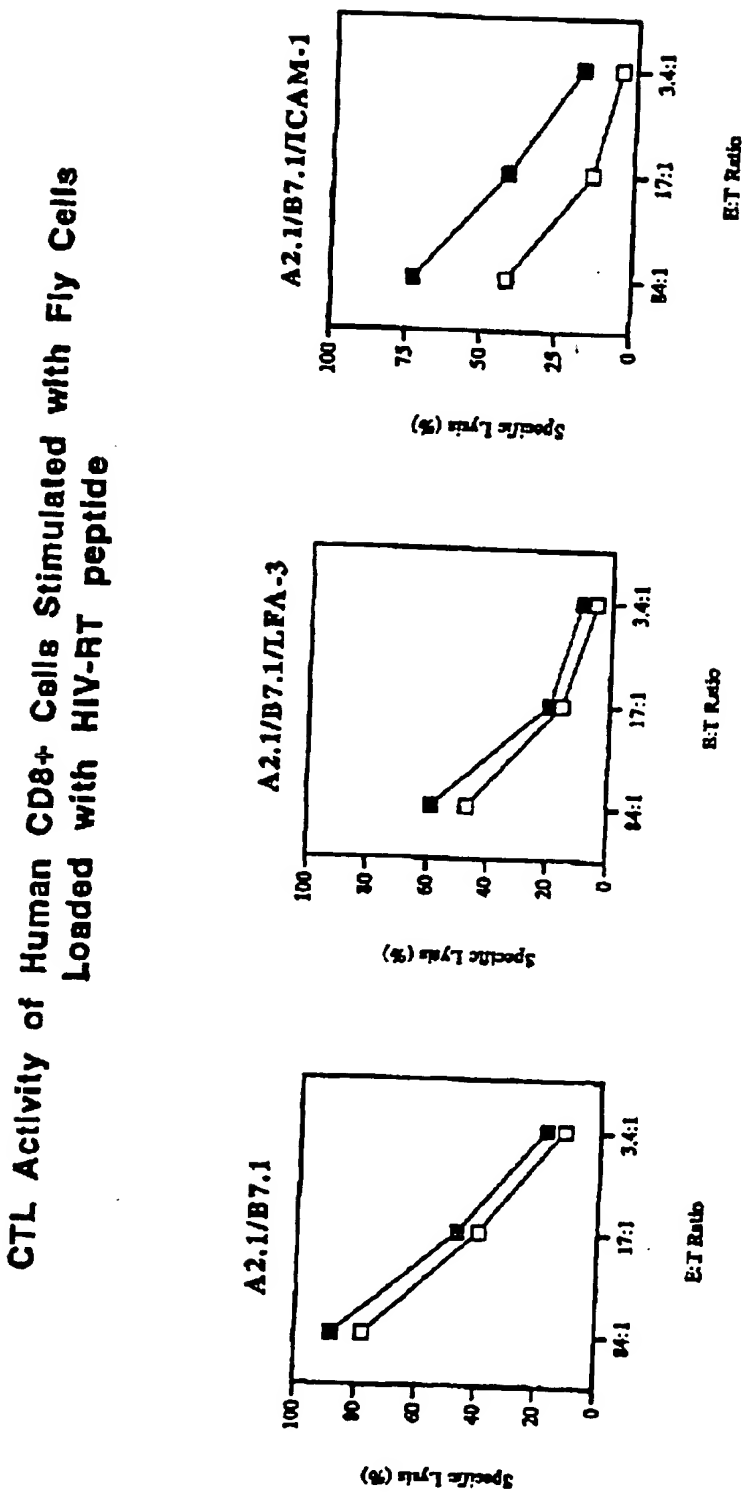
CTL Activity of Human CD8+ Cells Stimulated with Fly Cells Loaded with HIV-RT peptide
A2.1/B7.1
A2.1/B7.1/LFA-3
A2.1/B7.1/ICAM-1
Specific Lysis (%)
E:T Ratio
84:1
17:1
3.4:1
Target Cells: JY+Control Peptide
Target Cells: JY+RT

FIGURE 27

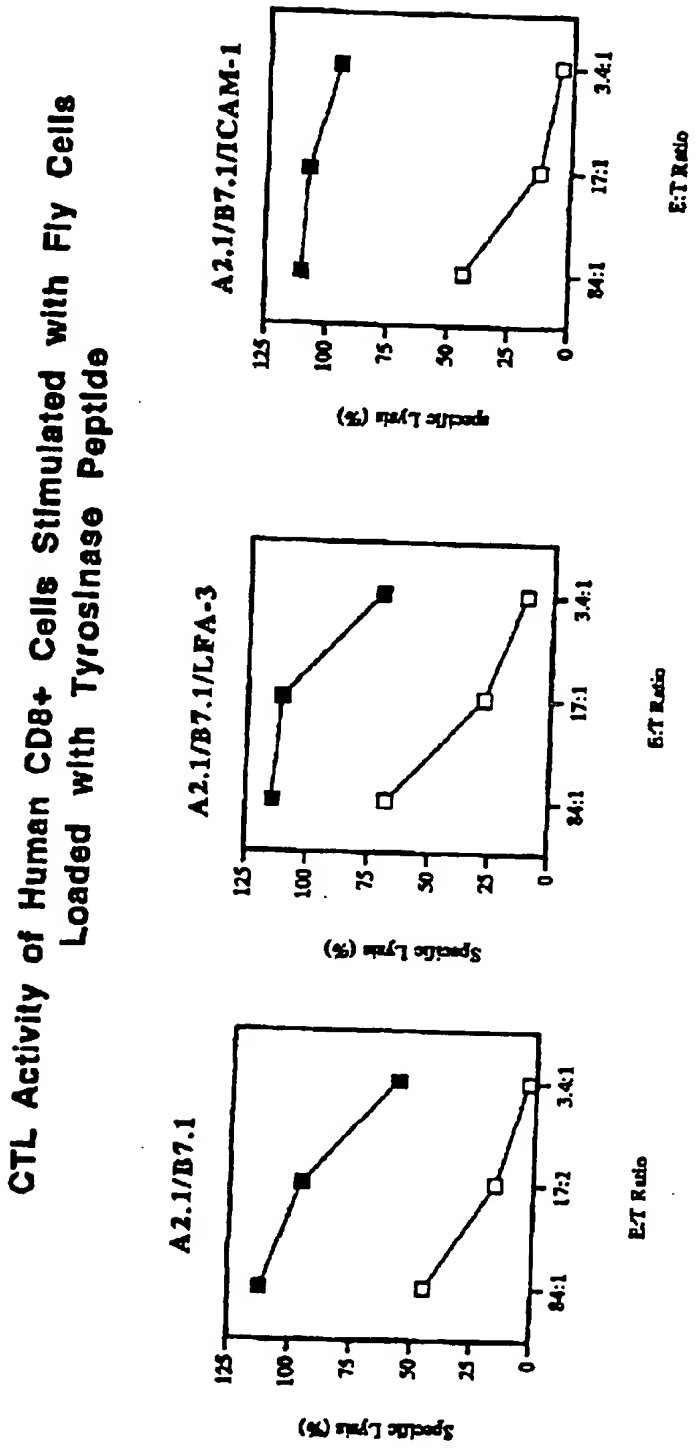
CTL Activity of Human CD8+ Cells Stimulated with Fly Cells Loaded with Tyrosinase Peptide
A2.1/B7.1
A2.1/B7.1/LFA-3
A2.1/B7.1/ICAM-1
Specific Lysis (%)
125
100
75
50
25
0
84:1
17:2
17:1
3.4:1
E:T Ratio
Target Cells: JY+ Control peptide
Target Cells: JY+ Tyr

FIGURE 28

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/03249

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 45/00; C07K 17/00; C12N 5/00

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.21, 195.11, 534, 435/69.3, 240.2; 530/403

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Automated Patent System (APS) and DIALOG (file = BIOCHEM) database searches. Key words: MHC?, matrix, ICAM?, B7, coligand, costim?, antigen(w)present?, T(w)cell.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,314,813 (PETERSON ET AL.) 24 May 1994, see entire document, especially claim 1.	1-68, 79-87.
Y	Nature, Volume 332, issued 10 March 1988, J. C. Madsen et al., "Immunological unresponsiveness induced by receipient cells transfected with donor MHC genes", pages 161-164, see entire article.	1-68, 79-87
Y	W. F. PAUL, FUNDAMENTAL IMMUNOLOGY, published 1993 by Raven Press (New York), pages 133-135, see pages 133-135.	1-68, 79-87

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 JUNE 1996	27 JUN 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer THOMAS CUNNINGHAM Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

Inte...ational application No.
PCT/US96/03249

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 69-78
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

These claims are missing in whole or in part because page 154 of the disclosure is blank and is denoted as "MISSING PAGE".

3. ☒ Claims Nos.: 53
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/03249

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/93.21, 195.11, 534; 435/69.3, 240.2, 252.3; 530/403; 536/23.5

SUPPLEMENTAL SHEET

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/03249

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 45/00; C07K 17/00; C12N 5/00

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.21, 195.11, 534, 435/69.3, 240.2; 530/403

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Automated Patent System (APS) and DIALOG (file = BIOCHEM) database searches. Key words: MHC?, matrix, ICAM?, B7, coligand, costim?, antigen(w)present?, T(w)cell.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,314,813 (PETERSON ET AL.) 24 May 1994, see entire document, especially claim 1.	1-87
Y	Nature, Volume 332, issued 10 March 1988, J. C. Madsen et al., "Immunological unresponsiveness induced by receipient cells transfected with donor MHC genes", pages 161-164, see entire article.	1-87
Y	W. F. PAUL, FUNDAMENTAL IMMUNOLOGY, published 1993 by Raven Press (New York), pages 133-135, see pages 133-135.	1-87

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 JUNE 1996	24 JUL 1996

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	THOMAS CUNNINGHAM
Facsimile No. :(703) 305-3230	Telephone No. :(703) 308-0196

Form PCT/ISA/210 second sheet(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/03249

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/93.21, 195.11, 534; 435/69.3, 240.2, 252.3; 530/403; 536/23.5